# INDUCTION OF HEAT SHOCK PROTEINS IN LIVER DURING HEPATOTOXICANT EXPOSURE

By

WILLIAM F. SALMINEN, JR.

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I dedicate this dissertation to my wife and three boys who supported me throughout my doctoral work. My wife worked three hours away, two days a week so that our boys could stay at home during their first years of life and I could work on my dissertation at the same time. Without her support this research would not have been possible. My three boys (Tyler, Alec, and Jake) gave me tremendous inspiration for my accomplishments. Besides, it's great when your son says, "My daddy is a scientist just like Bill Nye the science guy."

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### ABBREVIATIONS AND DEFINITIONS

ALT= Serum alanine aminotransferase activity

AMAP= 3'-Acetamidophenol

APAP= Acetaminophen

ßNF= Beta-naphthoflavone

BSO= L-Buthionine-[S,R]-sulfoximine

DEN= Diethylnitrosamine

DMN= Dimethylnitrosamine

HPLC= High performance liquid chromatography

HSE= Heat shock element

HSF= Heat shock transcription factor

HSP= Heat shock protein

LDH= Lactate dehydrogenase

PB= Phenobarbital

Proteotoxicity= Any stress or insult that alters the native state of a protein

sub-LHS= Sub-lethal heat shock

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Chairman: Stephen M. Roberts, Ph.D.
Major Department: Pharmacology and Therapeutics

Heat shock proteins (hsps) are ubiquitous proteins expressed in both prokaryotic and eukaryotic organisms. They are believed to play a role in maintaining protein homeostasis by trafficking and refolding proteins throughout the cell. In addition, elevated levels of hsps have been correlated with protection against a variety of stressors. Since many hepatotoxicants disrupt protein homeostasis, it was hypothesized that hsp induction might occur in the liver after hepatotoxicant exposure as a mechanism to help the liver cope with the toxicant-induced proteotoxicity. A series of studies was conducted to determine if hepatotoxicants induce hsps in mouse liver and if elevated levels of hsps provide protection against the toxicant-induced damage. A variety of toxicants were able to induce hsp25 and hsp70i, without affecting the level of hsp60, hsc70, or hsp90 *in vivo*. Similar results were obtained in the HepG2 cell line in which some toxicants were potent inducers of hsp70i while others were not. Elevated levels of hsps produced by amphetamine-induced hyperthermia *in vivo*, or mild heat treatment in the HepG2 cell line, were correlated with protection from some, but not all of the toxicants. The intralobular pattern of hsp25 and

hsp70i induction in mouse liver was determined immunohistochemically for several toxicants to see what cells induced hsps during toxicant exposure. For acetaminophen (APAP) and bromobenzene, hsp25 induction was predominantly on the periphery of the lesions, whereas hsp25 induction after cocaine or carbon tetrachloride exposure was uniform throughout the lesions with no induction observed on the periphery. Experiments were conducted *in vivo* to determine if protein adduction without concurrent toxicity was sufficient to trigger hsp induction. N-Acetyl-cysteine (NAC) prevented APAP hepatotoxicity, but did not significantly affect APAP arylation of protein. The NAC treatment did not inhibit APAP-induced hsp accumulation indicating that protein binding without concurrent toxicity may be sufficient to trigger hsp induction. These results show that some toxicants are potent inducers of hsps and that elevated levels of hsps may provide protection against some toxicants indicating that hsp induction during toxicant exposure might be a protective mechanism to deal with the toxicant-induced injury.

# CHAPTER 1
# BACKGROUND AND OBJECTIVES

## Background and Objectives

The experiments outlined in this dissertation were devised to test the hypothesis that hsp induction occurs in the liver after hepatotoxicant exposure as a result of the toxicant-induced proteotoxicity. In addition, some of the following experiments address the hypothesis that hsp induction during toxicant exposure is a protective mechanism to help the liver repair or eliminate toxicant-damaged proteins. Most of the results presented in this dissertation were obtained from experiments conducted in the whole animal. This is a unique aspect of this study since very few past investigations have looked at the role hsps play during stress in the whole animal. Through the use of a variety of experimental approaches *in vitro* and *in vivo* the previously mentioned hypotheses were tested and the data gathered are presented and interpreted in this dissertation.

The heat shock response was discovered over 30 years ago when a study by Ritossa (1962) noted a new heat-inducible puffing pattern along the salivary gland chromosomes of *Drosophila melanogaster*. The puffs corresponded to increased heat shock protein (hsp) gene transcription. Since this first discovery, many types of hsps have been discovered and grouped into families based on their apparent molecular weight and amino acid sequence relationships: small hsps, hsp60, hsp70, hsp90, and high molecular weight hsp families. As their name implies, cells induce hsps when they are exposed to mild hyperthermia; however, exposure to other types of stress can also cause hsp induction (Parsell and Lindquist, 1994; Voellmy, 1994; Welch, 1992). In addition, some hsps are synthesized at very low levels and only induced upon exposure to a stress or "stressor." In the liver of naive mice, for example, hsp90, hsc70, and hsp60 are expressed constitutively

at appreciable levels, very low levels of hsp 70i are observed, and hsp25 is virtually undetectable (Wilkinson and Pollard, 1993; Klemenz et al., 1993; Bardella et al., 1987). In animals subjected to thermal stress, levels of hsp70i and hsp25 are increased dramatically (Lu and Das, 1993; Blake et al., 1990).

Hsps are grouped under the broad category of stress proteins which includes glucose regulated proteins (grps), ubiquitin, metallothioneins, and heme oxygenases. All of these proteins share the common feature of being induced during stress. Grps are readily induced by glucose deprivation and metallothioneins are readily induced by exposure to heavy metals. However, induction of a stress protein is not necessarily restricted to one type of stress, and induction of several types of stress proteins may occur simultaneously. Hsps and grps can be induced by heavy metals such as cadmium, and grps are slightly induced in some cells during exposure to hyperthermia (Goering et al., 1993; Joslin et al., 1991). Often, only a subset of hsps are induced during exposure to a given stress and the magnitude of induction often varies among those hsps that are induced (Sanchez et al., 1992; Mirkes et al., 1994). For example, Wiegant et al. (1994) noted dramatic induction of hsp70i during treatment of rat hepatoma cells with cadmium with no change in the level of hsp60. In addition, some hsps, most notably hsp25 and hsp70i, appear to be induced to a greater extent during exposure to many types of stresses compared to other hsps (Bernelli-Zazzera et al., 1992; Goering et al., 1993; Van Laack et al., 1993; Lin et al., 1994; Roberts et al., 1996).

Several observations suggest that accumulation of non-native protein may trigger hsp induction. Overexpression of mammalian proteins unable to fold properly caused activation of hsp genes in bacteria (Goff and Goldberg, 1985), and the injection of chemically-denatured proteins into vertebrate cells increased stress protein synthesis while injection of the corresponding native protein did not (Ananthan et al., 1986). Indeed, many of the agents that induce hsps also bind, denature, and/or alter the redox status of proteins (Lee and Dewey, 1988; Lee and Hahn, 1988; Lumpkin et al., 1988; Ciocca et al., 1993;

Lin et al., 1994). A widely accepted scenario for induction of hsp70 by denatured proteins, as described by Morimoto et al. (1994), is diagrammed in figure 1-1. In a normal eukaryotic cell, hsp70 is constitutively bound to heat shock transcription factor (hsf). Upon protein denaturation, hsp70 releases hsf and binds denatured proteins for which it has a higher affinity (Rothman, 1989; Burel et al., 1992; Beckman et al., 1992). The released hsf forms a homotrimer and translocates to the nucleus where it binds the heat shock element (hse), which is a sequence of DNA within the promoter of hsp genes (Amin et al., 1988; Sarge et al., 1993). Binding of hsf to the hse increases the rate of hsp gene transcription (Fernandes et al., 1994). Once additional hsp70 is produced and/or denatured proteins are refolded by hsps, the level of free hsp70 increases to such a level that it can once again bind hsf, returning the rate of hsp gene transcription to constitutive levels (Baler et al, 1992).

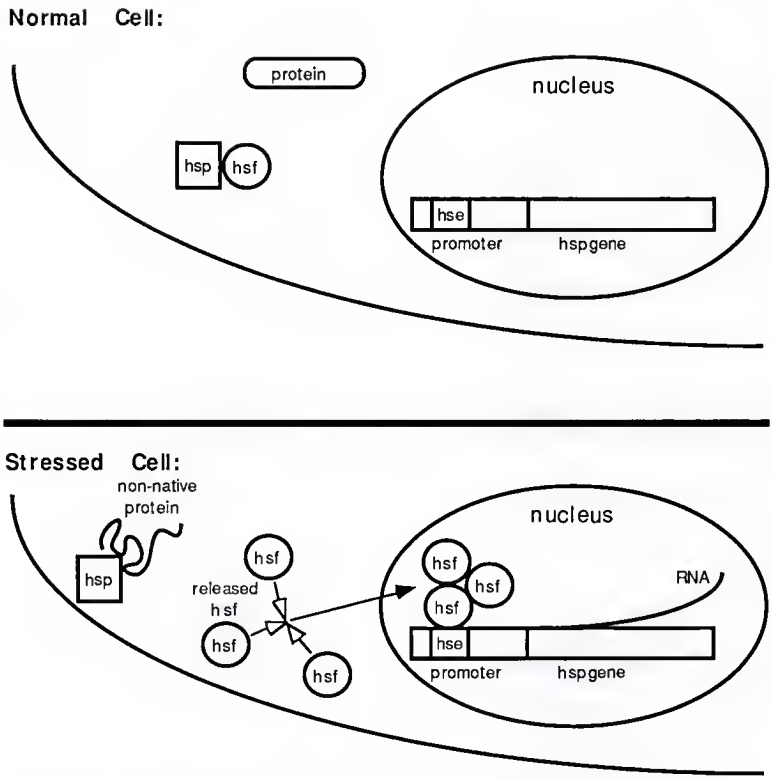



Figure 1-1. Mechanism of heat shock protein induction by denatured proteins.

Two other factors play a role in producing elevated levels of hsps during stress. In addition to the increased rate of hsp gene transcription, the hsp mRNA is preferentially translated and its half life is increased. During hyperthermia, the synthesis of normal proteins is down regulated while the translation of hsp mRNA is increased and the rate of hsp mRNA degradation is decreased (Theodorakis and Morimoto, 1987; Petersen and Lindquist, 1988; De Maio et al, 1993). It is through increased hsp gene transcription,

preferential translation of hsp mRNA, and stabilization of the hsp mRNA during stress that hsp accumulation occurs.

As mentioned above, hsp70 binds denatured proteins and releases the constitutively bound hsf. The binding of hsp70 to denatured proteins is believed to aid protein solubility by preventing the incorrect aggregation of exposed hydrophobic areas of non-native proteins (Beckman et al., 1992; Ciocca et al, 1993; Beck and De Maio, 1994). Hsp70 hydrolyzes ATP once the protein refolds to its native form using the energy derived from ATP hydrolysis to release the protein, not to actively refold the protein (Beckman et al., 1990). It is interesting to note that hsp70 also aids the translocation of proteins into the endoplasmic reticulum and mitochondria by binding hydrophobic areas of the proteins, keeping the proteins in a translocationally competent form (Chirico, 1992). Recent evidence also suggests that hsps may help target proteins for degradation if they are damaged beyond repair (Parag et al., 1987; Craig et al., 1994).

Many studies have correlated elevated levels of hsps with cytoprotection from a variety of stresses, the most notable being extreme hyperthermia (Hahn and Li, 1990; Feige and Mollenhauer, 1992). Recent studies have provided direct evidence that it is hsps, not a by-product of the inducing treatment, that provides cytoprotection. Li et al. (1991) transfected recombinant human hsp70 into rat fibroblasts and found that it protected the cells from thermal stress, and injection of antibodies against hsp70 into fibroblasts decreased their ability to survive short exposures of hyperthermia (Riabowol et al., 1988). The mechanism of hsp cytoprotection is believed to be due to the ability of hsps to bind denatured proteins and maintain their solubility until they can assume their native form. This process prevents protein precipitation and conserves valuable resources since protein can be recovered instead of degraded (Brown et al., 1993; Parsell and Lindquist, 1994). Most of the studies investigating the protective effect of elevated levels of hsps have been *in vitro* ; however, elevated levels of hsps have been shown to provide protection from a handful of stressors *n vivo* suggesting that the cytoprotective function of hsps is not unique

to cultured cells and is applicable to the whole animal (Villar et al., 1993; Hotchkiss et al., 1993; Currie et al., 1988; Saad et al., 1995).

Given the mechanism of action of hsps, it is reasonable to hypothesize that any stress that damages or denatures proteins should elicit a heat shock response. Indeed, many of the stimuli that induce hsps are known to alter the native form of proteins. Heavy metals such as cadmium and arsenic are strong inducers of hsps in many cell types and *in vivo* (Lee and Dewey, 1988; Goering et al., 1993; Bauman et al., 1993; Abe et al., 1994; Ovelgonne et al., 1994). These metals bind free sulfhydryl groups of proteins, altering their native state (Jacobson and Tumer, 1980). Many agents that organisms are exposed to alter the native form of proteins either directly or indirectly. Toxicants such as bromobenzene and carbon tetrachloride covalently bind protein which presumably denatures the target protein (Sipes and Gandolfi, 1982; Hanzlik et al, 1989). Toxicants may also alter protein conformation by altering the redox status of a cell. Diamide rapidly oxides reduced glutathione in a cell which leads to increased formation of protein-mixed disulfides (Grimm et al., 1985; Collison et al., 1986). It is conceivable that any xenobiotic that alters protein homeostasis should induce hsps.

The liver presents a unique model to investigate the role of the heat shock response during toxicant insult since the liver is the site of metabolism of the majority of xenobiotics. In mammals, the majority of cytochrome P450 mixed-function oxygenase activity is present in the liver along with the conjugating enzymes necessary to form toxicant metabolites that can be eliminated from the body (Plaa, 1993). Often, toxicants are metabolized to reactive intermediates that can overwhelm endogenous defense mechanisms ultimately resulting in cell damage (Lindamood, 1991). A typical example is acetaminophen (APAP) hepatotoxicity. Normally, a small fraction of the acetaminophen exposed to the liver is metabolized to the reactive N-acetyl-p-benzoquinoneimine (NAPQI) by cytochrome P450 mixed-function oxygenases. This metabolite is highly reactive and can bind cellular macromolecules; however, the metabolite is conjugated with glutathione

before it can attack nucleophilic cell molecules (Plaa, 1993). Only when glutathione levels are depleted does NAPQI bind cellular constituents and cause cell damage (Rashed et al., 1990).

Given the diversity of hepatotoxicants, it follows that cell damage occurs through a vast array of mechanisms in the liver. A few examples are lipid peroxidation, covalent binding to cell macromolecules, free radical production, inhibition of protein synthesis, and lipid accumulation (Marzella and Trump, 1991). The availability of a wide array of hepatotoxicants that cause damage through different mechanisms was employed in studies to help refine what role(s) hsps play during toxicant exposure in the liver. A variety of approaches and models were used to determine if hsps were able to recognize and alleviate toxicant induced damage in the liver.

The following experiments were devised to help determine what role hsps play in the liver during toxicant exposure. First, Western blot analysis of hsp levels in HepG2 cells and in mouse liver after toxicant exposure was used to determine whether or not hsps were induced by toxicants. Second, the pattern of hsp accumulation in mouse liver after toxicant exposure was determined immunohistochemically to detect which cells in the liver (i.e., normal or damaged) induced hsps after toxicant exposure. Third, the correlation between elevated levels of hsps and protection against hepatotoxicty was measured in HepG2 cells and in the whole animal to try and determine if elevated levels of hsps could provide protection against toxicant-induced damage. Finally, the ability of toxicant adducted protein to trigger hsp induction was measured in the whole animal to try and determine if toxicant adducted protein alone could trigger hsp induction independent of toxicity. The following results provide strong evidence that toxicants are able to induce hsps in liver, toxicant adduction of protein plays a major role in triggering hsp induction, and elevated levels of hsps may provide protection against some toxicants.

# CHAPTER 2
# INDUCTION OF HSP70 IN HEPG2 CELLS IN RESPONSE TO HEPATOTOXICANTS

## Introduction

Prokaryotic and eukaryotic cells respond to a variety of stresses by enhancing the transcription of a specific set of genes that encode heat shock proteins (hsp). In eukaryotic cells, increased levels of hsps occur as a result of the activation of a heat shock transcription factor (HSF) that is normally in an inactive form (reviewed by Voellmy, 1994), apparently as a heterooligomer with hsp70 (Baler, 1992; Wu et al., 1994). Stresses including heat shock causes protein unfolding and nonnative proteins have a higher affinity for hsp70 than native proteins (Flynn et al., 1989). Titration of hsp70 through such binding may cause the release of hsp70 from HSF, allowing HSF to assemble DNA binding homotrimers (Baler et al., 1992a; Westwood et al., 1992). The HSF homotrimers bind to promoters of hsp genes, and after a further stress-induced activation step become competent to enhance the transcription of the genes (Zuo et al., 1995). After removal of the stress, HSF returns to an inactive state, and transcription of hsp genes decreases at a rate that depends on the severity of the stress (Zuo et al., 1995).

The binding of hsp70 to damaged proteins is believed to assist in preventing their aggregation and promoting correct refolding ("molecular chaperoning"), as well as facilitating their degradation (Parsell and Lindquist, 1994). As such, hsp70 and other stress proteins represent an important mechanism by which cells prevent accumulation of aberrant proteins. Further, experimental manipulations that result in elevated levels of hsps (e.g., thermal treatment or use of an inducible expression vector) have been observed to result in cytoprotection from a variety of subsequent stresses such as a lethal heat shock (Li et al., 1990; Kampinga et al., 1995; Parsell and Lindquist, 1994). These observations

suggest that induction of hsp70 and other hsps may represent an important cellular defense mechanism against proteotoxicity from a variety of stressors.

Among the stressors that have been demonstrated to result in hsp induction is a rather extensive list of chemical toxicants (Goering et al., 1993; Nover, 1991; Levinson et al., 1980). The number and variety of toxicants shown, in one experimental system or another, to result in increased expression of hsps have led some to suggest that hsp induction may be a universal response to cytotoxicity (e.g., Sanders et al., 1993; Anderson et al., 1987; Blom et al., 1992). However, a recent study of stress protein induction in rat hepatoma cells in response to four chemical agents (viz., arsenite, cadmium, dinitrophenol, and ethanol) found marked differences among these agents in the ability to induce specific stress proteins. In fact, cytotoxic concentrations of two of these toxicants (dinitrophenol and ethanol) failed to induce any of the battery of hsps examined. The results of this study suggest that hsp induction in response to chemical toxicants may be more complex than originally envisioned, with some, but not all, toxicants producing proteotoxicity triggering an hsp response. Further, though not explicitly addressed in this study, it may be possible that the cytoprotection against chemical toxicants afforded by hsp induction observed in some studies (e.g., Ovelgonne et al., 1995; Kampinga et al., 1995; Steels et al., 1992) may not be applicable to all toxicants.

In the present study, we have examined the inducible 70-kDa hsp (hsp 70i) response to a variety of hepatotoxic agents in HepG2 cells. The HepG2 cell line, derived from a human liver hepatoma, was chosen as the model system since it is reported to retain many of the properties of primary cells, including the ability to metabolize a wide variety of toxicants (Doostdar et al., 1993; Neuman et al., 1993). This aspect was regarded as particularly important since most hepatotoxicants require bioactivation to produce their characteristic effects on the liver. Also, HepG2 cells have been shown to display the classical heat shock response, i.e. mild hyperthermia leads to induction of hsps, and the resulting elevated levels of hsps provide protection from subsequent severe hyperthermic

treatment (De Maio et al., 1993). Using this model system, the effect of each hepatotoxicant on hsp 70i expression was evaluated. Additionally, the ability of elevated levels of hsps to afford cytoprotection from each hepatotoxicant was examined and compared with its ability to induce hsps.

## Materials and Methods

Cell culture and treatments. HepG2 cells, obtained from the American Type Culture Collection (ATCC No. HB 8065, Rockville, Maryland), were cultured in Earle's salt-based MEM supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, Utah), 1 mM sodium pyruvate, and 50 mg/L gentamicin in a humidified, 5% $CO_2$ atmosphere maintained at 37° C. Cells were grown to confluency in 60 x 15 mm tissue culture plates or T25 tissue culture flasks (Corning Glass Works, Corning, NY) before all treatments and assays. The T25 flasks were used for carbon tetrachloride or bromobenzene exposure so that the volatile compounds did not escape the flasks. In some experiments, cells were made hyperthermic by floating plates of cells in a circulating water bath maintained at the desired temperature within 0.1° C. Sub-lethal heat shock was induced by increasing the culture medium temperature to 43° C for one hour. Lethal heat shock was produced by increasing the culture medium temperature to 46.5° C for two hours. After heat treatment the culture medium was replaced and the plates and/or flasks returned to the incubator. Cadmium acetate, cocaine hydrochloride, cyclophosphamide monohydrate, or N-nitrosodiethylamine (diethylnitrosamine) (Sigma Chemical Co., St. Louis, MO) were dissolved in culture medium to the desired concentration. The culture medium was then sterile-filtered before being placed on the cells. Preliminary experiments found that adding bromobenzene or carbon tetrachloride to the culture medium resulted in at least a fivefold decrease in medium concentration over the first 30 minutes as these volatile agents evaporated into the headspace of the flask. Increasing the amount of carbon tetrachloride or bromobenzene added to the flask to achieve the desired final concentration resulted in

unacceptably high, transient concentrations of toxicant. To avoid this problem, bromobenzene or carbon tetrachloride were introduced to the culture medium by placing a measured amount into a 9 mm diameter x 3 mm height polypropylene container that was placed on the sloping edge of a T25 flask containing 3 mls of culture medium and sealing the flask. Pilot studies found that both toxicants quickly volatilized from the vessel, and an equilibrium between the headspace and culture medium was achieved within 30 minutes. A series of experiments were conducted to determine the appropriate amounts to add to the vessel to achieve the desired concentrations in the culture medium. With the flask sealed, the concentration of carbon tetrachloride or bromobenzene in the culture medium decreased no more than 25% over 24 hours at 37° C.

Gas chromatography. To measure carbon tetrachloride and bromobenzene concentrations in the culture medium, one milliliter of culture medium was added to 2 mls of pentane. One hundred microliters of a 20 µg/ml trichloroethylene solution was added to each sample to serve as an internal standard. The mixture was vortexed for one minute and centrifuged at 3,000 x g at 5° C for ten minutes to separate the layers. A 3 µl aliquot of the top (pentane) layer was injected onto an Econo-cap SE-54 column, 15 m x 0.54 mm ID (Alltech, Deerfield, IL). The following conditions were used: helium carrier gas flow rate of 5 ml/min.; nitrogen makeup gas flow rate of 50 ml/min.; injector port temperature was 110°C, and detector temperature was 200°C. The oven temperature was 50°C for carbon tetrachloride analysis and 120°C for bromobenzene analysis. The eluted compounds were detected by an electron capture detector. Carbon tetrachloride and bromobenzene were quantitated using standard curves prepared from culture medium spiked with known amounts of reference compound.

Polyacrylamide gel electrophoresis. Cells were homogenized in sample buffer (0.05 M Tris[hydroxymethyl]aminomethane (Tris-HCl, pH 6.8), 2% sodium dodecyl sulfate, 10 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride). Each sample was boiled for five minutes, passed through a 22 ga. needle three times to sheer DNA, and

stored at -80°C until use. Twenty micrograms of protein from each sample was aliquoted to separate tubes, and bromphenol blue was added to a final concentration of 0.0025%. Each aliquot was boiled for five minutes, loaded onto separate lanes of a 10% SDS-PAGE gel, and resolved by electrophoresis (Laemmli, 1970).

Protein blotting and immunostaining. Proteins separated by SDS-PAGE were immediately blotted to supported nitrocellulose (Bio-Rad Laboratories, Hercules, CA) using a semi-dry blotting apparatus (Millipore, Bedford, MA) and one-half strength Towbin buffer (10 mM Tris-base, 96 mM glycine, and 10% methanol). Blotting was done at 320 mA for 1.5 hours. Upon completion, the membrane was blocked in TBS (20 mM Tris-HCl, pH 7.5, 500 mM sodium chloride) containing 3% gelatin. The membrane was washed two times for five minutes each in TTBS (TBS containing 0.05% polyoxyethylenesorbitan monolaurate) and probed with an antibody specific for hsp 70i (Stressgen, Victoria, BC, Canada) at a 1:1000 dilution in TTBS containing 1% gelatin. Incubation was for 18 hours at 24°C with continuous shaking. The membrane was washed two times for five minutes each with TTBS and then a goat anti-mouse alkaline phosphatase-conjugated antibody (Bio-Rad Laboratories, Hercules, CA) at a 1:3000 dilution in TTBS containing 1% gelatin was incubated with the membrane for one hour at 24°C with continuous shaking. Next the membrane was washed two times for five minutes each in TTBS and once for five minutes in TBS. The colorimetric substrate BCIP/NBT (100 mM Tris-base, pH 9.5, 165 µg/ml 5-bromo-4-chloro-3-indolyl phosphate, 330 µg/ml nitro blue tetrazolium, 100 mM sodium chloride, 5 mM magnesium chloride) was added to the membrane to localize antibody binding.

Lactate dehydrogenase (LDH) leakage assay. Two hundred microliters of culture medium from each plate of cells was removed and the amount of LDH activity present determined spectrophotometrically using the LD-L assay (Sigma Chemical Co., St. Louis, MO). All samples were analyzed at 340 nm using a kinetic microplate reader (Molecular Devices, Menlo Park, CA). The cells were then lysed by adding Triton X-100 to the culture

medium to a final concentration of 0.7%. Two hundred microliters of the culture medium containing the lysed cells was removed and the amount of LDH activity determined. The percent of LDH released into the culture medium during incubation was determined by dividing the culture medium-only LDH activity by the total LDH activity (i.e. after cell lysis). The percent of LDH leakage was used as an indicator of cell viability.

HSP 70i mRNA analysis. Total RNA was isolated from treated cells using the acid guanidinium-phenol-chloroform method as described previously (Chomczynski and Sacchi, 1987). Twenty micrograms of RNA from each sample was denatured in 6.5% formaldehyde, 1X MOPS running buffer (0.02 M 3-[N-morpholino]propanesulfonic acid, 8 mM sodium acetate, 1 mM [ethylenedinitrilo]tetraacetic acid), and 50% formamide by heating at 65°C for 15 minutes and then cooling on ice. Loading buffer was added to each sample to a final concentration of 5% glycerol, 0.1 mM EDTA (pH 8.0), and 0.025% bromphenol blue and loaded onto separate lanes of a 1.2% agarose/ 1X MOPS running buffer/ 6.6% formaldehyde gel. After resolving the RNA by electrophoresis, the RNA was transferred to a positively charged nylon membrane by capillary transfer using 20X SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0). RNA was fixed to the membrane using 120 mJ/cm$^2$ of 254 nm UV light. Prehybridization was performed using 5X SSC, 50% formamide, 0.02% sodium dodecyl sulfate, 0.1% N-lauroylsarcosine, and 2% blocking reagent (Genius System, Boehringer Mannheim, USA) at 42°C for two hours. Hybridization was carried out in freshly prepared prehybridization solution containing 20 ng/ml of a digoxigenin-labeled hsp 70i DNA (ATCC No. 57494, American Type Culture Collection, Rockville, Maryland). Hybridization was at 42°C for 18 hours. The membrane was washed in 2X SSC, 0.1% sodium dodecyl sulfate, pH 7.0 at 24°C and then in 0.5X SSC, 0.1% sodium dodecyl sulfate, pH 7.0 at 65°C. Detection of bound probe was accomplished with an alkaline phosphatase-conjugated anti-digoxigenin antibody and the chemiluminescent substrate Lumiphos 530 (Boehringer Mannheim, USA) according to the manufacturer's directions. Luminescence was detected using standard X-ray film. To

ensure equal transfer of the RNA to the membrane, the membrane was stripped by boiling in 0.1% SDS and reprobed using a digoxigenin-labeled ß-actin cDNA (ATCC No. 65128, American Type Culture Collection, Rockville, Maryland).

Covalent binding. [$^{14}$C]-Carbon tetrachloride (New England Nuclear, Boston, MA), ring-labeled [$^{14}$C]-bromobenzene (ICN Radiochemicals, Irvine, CA) or tropine ring-labeled [$^{3}$H]-cocaine hydrochloride (National Institute on Drug Abuse, Rockville, MD) were used to measure covalent binding of toxicant to proteins. Radiolabeled toxicant was added to unlabeled toxicant such that each culture plate received 1 µCi at the desired total toxicant concentration (5 mM for cocaine, 0.8 mM for carbon tetrachloride, and 1.5 mM in the case of bromobenzene). As a control, incidental binding was measured for each radiolabeled toxicant using cells pretreated with trichloroacetic acid (6% w/v) to inhibit metabolism. After exposure, cells were scraped from the plate, transferred to a 15 ml conical tube, and centrifuged at 2000 x g for five minutes. The cell pellet was rinsed with phosphate buffered saline (10 mM potassium phosphate, 2.7 mM potassium chloride, 120 mM sodium chloride, pH 7.4) and the protein precipitated with 1 ml of 6% trichloroacetic acid (w/v). The solution was transferred to a 1.5 ml centrifuge tube, and protein was pelleted by centrifugation at 14000 x g for five minutes. The pellet was washed extensively with 1 ml aliquots of methanol/ether (3:1) until [$^{14}$C]- or [$^{3}$H]-radioactivity in one-half volume of the supernatant was indistinguishable from background. The pellets were air dried and resuspended in 1 N sodium hydroxide. [$^{14}$C]- or [$^{3}$H]-Radioactivity in each sample was detected by liquid scintillation spectrometry. The protein concentration of each sample was measured as described below and used to normalize the detected radioactivity to the protein content.

Protein determination. Protein concentration was measured by the method of Bradford (Bradford, 1976) using the Micro Protein Determination assay (Sigma Chemical Co., St. Louis, MO). Bovine serum albumin was used as standard.

Statistical Analysis. Data were analyzed by a one-way ANOVA followed by a Student Neuman-Keuls post-hoc test. The level of significant difference was defined as the 0.05 level of probability.

## Results

In initial experiments, the time course of hsp 70i induction in HepG2 cells was determined using the classical hsp inducer, sub-lethal heat shock (sub-LHS; 43°C for 1 hour) (see Figure 2-1). Induction of hsp 70i, as determined by Western blotting, could be seen as early as one hour post sub-LHS with maximal accumulation occurring at 24 hours. Although somewhat diminished, Hsp 70i was still substantially elevated above control levels at 72 hours post sub-LHS. Based on this information, the time to peak accumulation, 24 hours, was chosen as the best interval to evaluate the presence of hsp 70i induction after toxicant exposure.

The extent of induced accumulation of hsp 70i after hepatotoxicant exposure is shown in Figure 2-2. The hsp 70i signal on Western blots after hepatotoxicant exposure was quantitated using scanning densitometry and compared with the signal produced by untreated cells. In this experiment, hepatotoxicant concentrations were selected, as determined by LDH leakage, to produce approximately 40-70% cell mortality within 24 hrs. Under these conditions, diethylnitrosamine (50 mM), cadmium acetate (50 μM), bromobenzene (1.5 mM), and cyclophosphamide (20 mM) significantly induced the level of hsp 70i, while carbon tetrachloride (0.8 mM) and cocaine (5 mM) produced no change in the level of hsp 70i compared to untreated cells. Lower concentrations of each hepatotoxicant (concentrations that caused 8- 15% cell mortality) were also tested to ensure that hsp 70i induction was not being inhibited by excessive cytotoxicity in some cases. Cadmium acetate was the only hepatotoxicant with greater hsp 70i expression at the lower concentration. The other hepatotoxicants displayed no detectable hsp 70i induction at the reduced concentration (data not shown).

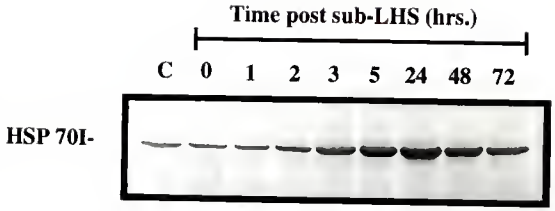



Figure 2-1. Immunochemical detection of hsp 70i in HepG2 cells at various times after a sub-lethal heat shock (43°C for one hour). Protein was resolved on a 10% SDS-PAGE gel, and hsp 70i was detected by Western blotting using a monoclonal antibody specific for hsp 70i. Equal amounts of protein (i.e., 50 μg) from each sample were loaded onto separate lanes. Control cells were maintained at 37°C.

A further set of experiments was conducted to determine whether the apparent absence of an hsp 70i induction response to carbon tetrachloride and cocaine was due to an inhibition of transcription. The levels of hsp 70i mRNA in HepG2 cells after treatment with the hepatotoxicants for 24 hours were measured by Northern blotting (Figure 2-3). Consistent with measurements of protein expression, diethylnitrosamine, cadmium acetate, bromobenzene, and cyclophosphamide all induced the level of hsp 70i mRNA, while carbon tetrachloride and cocaine produced no change. These observations suggest that the absence of an hsp 70i response to carbon tetrachloride and cocaine is not due to inhibition at the transcriptional level.

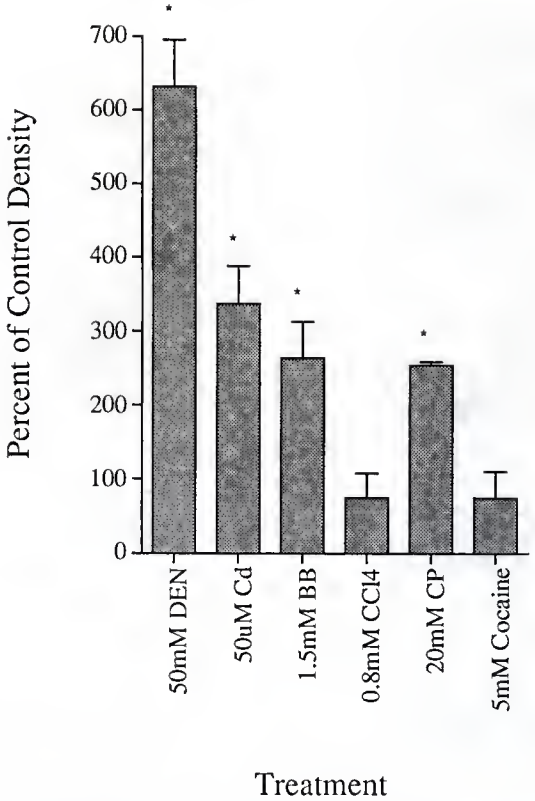



Figure 2-2. Scanning densitometry analysis of the level of hsp 70i in HepG2 cells 24 hours after hepatotoxicant treatment. Cells were exposed for 24 hours to various hepatotoxicants at the indicated concentrations. Protein was resolved on a 10% SDS-PAGE gel, and hsp 70i detected by Western blotting using a monoclonal antibody specific for hsp 70i. A goat anti-mouse IgG antibody conjugated with alkaline phosphatase was used to detect the primary antibody binding. DEN= diethylnitrosamine; Cd= cadmium acetate; BB= bromobenzene; $CCl_4$= carbon tetrachloride; CP= cyclophosphamide. Values represent mean ± SEM (n=3 plates). * denotes significantly different from control by Student Neuman-Keuls post-hoc test using $p<0.05$.

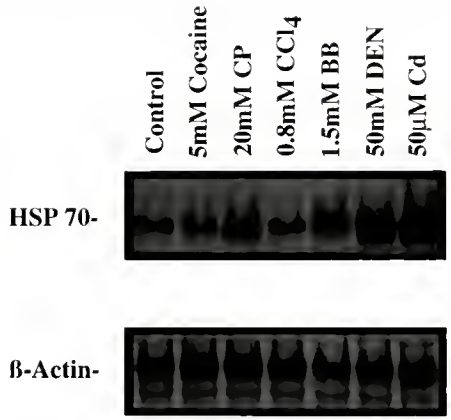



Figure 2-3. Northern blot showing the level of hsp 70i mRNA in HepG2 cells 24 hours after hepatotoxicant treatment. Cells were exposed for 24 hours to various hepatotoxicants at the indicated concentrations. Total RNA was isolated, resolved and blotted as described in Methods. Hsp 70i mRNA was detected by hybridizing the immobilized RNA with digoxigenin-labeled hsp 70i DNA. Hybridizing probe was located using an anti-digoxigenin antibody and a chemiluminescent substrate. The blot was stripped and reprobed with a digoxigenin-labeled ß-actin probe. Sub-LHS= sub-lethal heat shock (43°C for one hour); DEN= diethylnitrosamine; Cd= cadmium acetate; BB= bromobenzene; $CCl_4$= carbon tetrachloride; CP= cyclophosphamide.

Previous studies have shown that exposure to a sub-LHS may provide protection from other types of subsequent stresses. It has been inferred, but not shown, that the induction of hsps is responsible for this effect. It is possible that the induction of hsp 70i during exposure to some of the hepatotoxicants tested is a cytoprotective response of the cell; however, it may also be a by-product of cellular damage and serve no cytoprotective function. To examine these possibilities, the effect of a sub-LHS on hepatotoxicant potency was measured. Cells were subjected to sub-LHS, followed 24 hours later by

exposure to hepatotoxicant. The effect of sub-LHS on hepatotoxicant-induced cell lethality, as measured by LDH leakage, is shown in Figures 2-4 and 2-5. As a positive control, sub-LHS pretreatment diminished the cell mortality from lethal heat shock (46.5°C for two hours) exposure, as expected. Sub-LHS also significantly diminished the cytolethality of diethylnitrosamine, cadmium acetate, bromobenzene, and cyclophosphamide (Figures 2-4 and 2-5). Interestingly, sub-LHS pretreatment caused no significant change in the hepatotoxicant-induced cell lethality from carbon tetrachloride or cocaine (Figure 2-4).

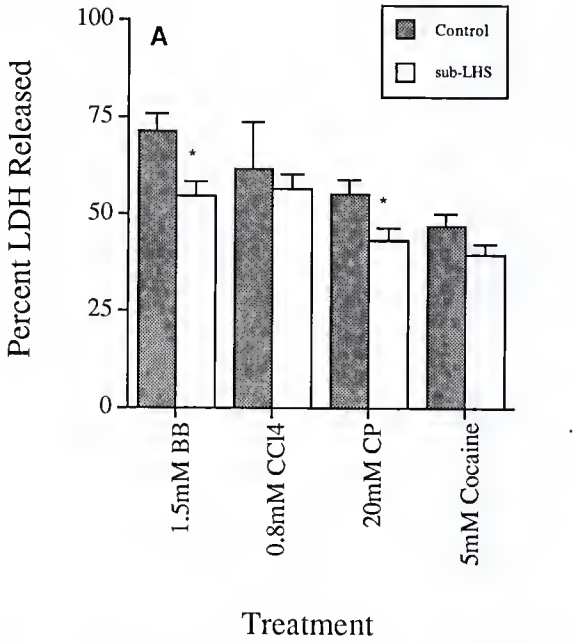



Figure 2-4. The effect of a 24 hour prior sub-lethal heat shock (sub-LHS= 43°C for one hour) on hepatotoxicant-induced cell lethality. Cells were heated in a water bath maintained at 43°C ± 0.1°C. Twenty four hours after the sub-LHS, hepatotoxicants were incubated with the cells for an additional 24 hours and the percent of LDH activity released into the culture medium was determined. BB= bromobenzene; $CCl_4$= carbon tetrachloride; CP= cyclophosphamide. Values represent mean ± SEM (n=5 plates). * denotes significantly different from control by Student Neuman-Keuls post-hoc test using $p<0.05$.

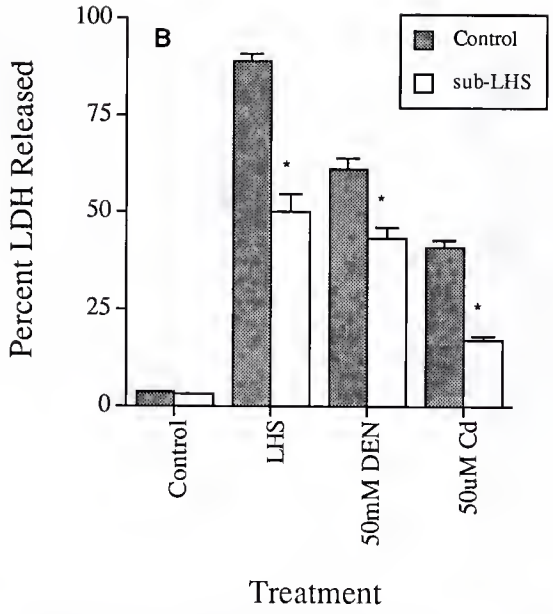



Figure 2-5. The effect of a 24 hour prior sub-lethal heat shock (sub-LHS= 43°C for one hour) on hepatotoxicant-induced cell lethality. Cells were heated in a water bath maintained at 43°C ± 0.1°C. Twenty four hours after the sub-LHS, hepatotoxicants were incubated with the cells for an additional 24 hours and the percent of LDH activity released into the culture medium was determined. LHS= lethal heat shock (46.5°C for two hours); DEN= diethylnitrosamine; Cd= cadmium acetate. Values represent mean ± SEM (n=5 plates). * denotes significantly different from control by Student Neuman-Keuls post-hoc test using $p<0.05$.

One mechanism by which the hepatotoxicants in this study might stimulate hsp 70i induction is through adduction of proteins by reactive metabolites. Among the hepatotoxicants tested, carbon tetrachloride, cocaine, diethylnitrosamine, cyclophosphamide, and bromobenzene each produce reactive metabolites in vivo (Evans, 1983; Sipes and Gandolfi, 1982; Hanzlik et al., 1989; Kanekal et al., 1992; Osterman-Golkar and Bergmark, 1988; Plaa, 1993). In order to interpret the apparent absence of hsp

70i induction by cocaine and carbon tetrachloride in HepG2 cells in the context of this potential mechanism, it was important to establish whether or not reactive metabolites were, or were not, being formed under the incubation conditions employed. To test this, the formation of reactive metabolites was evaluated through measurement of covalent (i.e., irreversible) binding to proteins following incubation with radiolabeled $[^{14}]$C-carbon tetrachloride, $[^{14}]$C-bromobenzene, or $[^{3}]$H-cocaine. As shown in Table 2-1, among these three hepatotoxicants, only bromobenzene produced detectable binding to protein.

Table 2-1. Irreversible Binding of Bromobenzene, Carbon Tetrachloride, or Cocaine to Protein in HepG2 Cells.

| Treatment | Duration (hours) | pmoles bound per μg protein |
|---|---|---|
| 1.5 mM Bromobenzene | 4 | 13.36 ± 6.66 |
| | 24 | 20.78 ± 5.67 |
| 0.8 mM Carbon tetrachloride | 4 | ND |
| | 24 | ND |
| 5 mM Cocaine | 4 | ND |
| | 24 | ND |

Note. HepG2 cells were treated with [$^{14}$C]-bromobenzene, [$^{14}$C]-carbon tetrachloride, or [$^{3}$H]-cocaine (1 μCi/plate) at the indicated concentrations. As a control, incidental binding was measured using the same exposure regimen in cells pretreated with trichloroacetic acid (6% w/v). Cells were harvested at the indicated times and washed extensively as described in Methods. ND= no detectable irreversible binding observed. Values represent mean ± SEM (n=3 plates) after subtracting incidental binding.

## Discussion

A common theme among agents that induce the heat shock response is the ability to disrupt protein homeostasis (Ananthan et al., 1986; Hightower, 1991). Many studies have focused on the induction of hsp 70i since it appears to be universally induced during stress. Consistent with recent reports by several investigators, however, the present study

suggests there are exceptions to this rule, even though cell injury occurs (Wiegant et al., 1994; Mirkes et al., 1994; Goodman and Sloviter, 1993).

A strong correlation between hepatotoxicant induction of hsp 70i and the ability of sub-LHS pretreatment to provide protection from those hepatotoxicants was observed. Diethylnitrosamine, cadmium acetate, bromobenzene, and cyclophosphamide induced hsp 70i and showed decreased cytotoxicity in cells pretreated with sub-LHS. In contrast, no change in the level of hsp 70i was evident after either carbon tetrachloride or cocaine treatment, nor was their cytotoxicity diminished in cells pretreated with sub-LHS. In this study, the extent of hsp 70i induction was evaluated at only one time point, and the relative ability of the various hepatotoxicants to increase hsp 70i levels cannot therefore be determined with confidence. To the extent that the 24 hour data reflect overall induction, however, the magnitude of induction of hsp 70i appears to be correlated with the ability of a prior sub-LHS to provide protection from the hepatotoxicants. Diethylnitrosamine and cadmium strongly induced hsp 70i and showed the greatest decrease in cytotoxicity in cells given a prior sub-LHS. Cyclophosphamide and bromobenzene mildly induced hsp 70i at 24 hours and showed only minimal decreases in cytotoxicity. The apparent correlation between the ability of a hepatotoxicant to increase hsp 70i levels and cytoprotection afforded by elevated levels of hsps argues that hsp 70i may play an important role in providing cytoprotection from hepatotoxicants. Information regarding similar correlations for other toxicants is extremely limited. Li and coworkers, however, in a study of hsp induction and cytotoxicity of a series of membrane-active agents (viz., solvents and local anesthetics), also found a strong correlation between the ability to stimulate hsp synthesis and cytoprotection afforded by prior hsp induction in Chinese hamster cells (Hahn et al., 1985).

Since carbon tetrachloride and cocaine did not induce hsp 70i and showed no decrease in cytotoxicity in cells pretreated with sub-LHS, it was necessary to investigate the possibility that the concentrations of toxicants used may have precluded the expression of

hsp 70i through inhibition of transcription, translation or some other means. To address these questions, Northern blots measuring hsp 70i mRNA were used to determine if transcription of the hsp 70i gene was inhibited by the toxicants. In addition, the effect of lower hepatotoxicant concentrations on the level of hsp 70i was measured. Neither of these experimental approaches indicated that carbon tetrachloride or cocaine were preventing the expression of hsp 70i at the concentrations used. The levels of hsp 70i mRNA after carbon tetrachloride or cocaine treatment were, in fact, the same as in untreated cells. These observations suggest that either carbon tetrachloride and cocaine are not producing cellular changes triggering hsp induction (in contrast to the other toxicants), or that they are inhibiting expression prior to, or at a level of, transcription of the hsp 70i gene.

Induction of hsps is believed to be triggered by denatured proteins (Ananthan et al., 1986; Baler et al., 1992). Many hepatotoxicants are metabolized to reactive intermediates that bind cellular proteins, and it is possible that the adducted proteins are recognized by hsps as nonnative, thereby triggering induction. This type of mechanism has been proposed by Chen et al. (1992) for hsp induction by nephrotoxic cysteine conjugates. Among the toxicants tested in the present study, all are capable of producing reactive metabolites which bind to protein in vivo, with the exception of cadmium, which can bind to proteins directly through interaction with sulfhydryls (Jacobson and Turner, 1980). Assuming that each of the toxicants also bound to protein in the HepG2 model system, the absence of hsp 70i induction following exposure of HepG2 cells to cytotoxic concentrations of carbon tetrachloride and cocaine appeared initially to argue against a reactive metabolite binding mechanism. Subsequent experiments, however, served to reinforce a correlation between covalent binding and hsp induction. Though HepG2 cells have been reported to metabolize many toxicants through cytochrome P-450 dependent mixed function oxidation (Belisario et al., 1991), reactive metabolite binding was found to be absent in the HepG2 cells during the carbon tetrachloride and cocaine exposures. Bromobenzene concentrations that induced hsp 70i expression in the HepG2 cells, on the

other hand, resulted in readily detectable covalent binding to proteins. Thus, HepG2 cells may be incapable of bioactivating carbon tetrachloride and cocaine. It is worthwhile noting that in mice, where administration of cocaine or carbon tetrachloride results in significant covalent binding, hsp 70i induction has been observed (unpublished observations).

In conclusion, experiments conducted using the HepG2 human hepatoma cell line and a battery of hepatotoxicant chemicals indicate that cytotoxicity is not always accompanied by hsp 70i induction. Most of the toxicants tested produced at least moderate induction, but some appeared to be completely ineffective. It is logical to suspect that differences in induction reflect differences in interactions between the toxicants and cellular proteins, such as through the formation of reactive metabolites, although this has not yet been clearly demonstrated. Perhaps one of the most interesting observations was the strong apparent correlation between the ability of the hepatotoxicants to induce hsp 70i and the extent of cytoprotection against their effects afforded by sub-LHS pretreatment. This suggests that, at least for some hepatotoxicants, hsp 70i induction may be an important cellular defense mechanism.

# CHAPTER 3
# HEAT SHOCK PROTEIN INDUCTION IN MURINE LIVER AFTER ACUTE TREATMENT WITH COCAINE

## Introduction

Heat shock proteins (hsps) are ubiquitous in nature and can be grouped into a number of distinct families based on size and amino acid sequence relationships. Most widely recognized are the hsp90, hsp70, hsp60, and hsp25 families. Several families include members that are constitutively expressed as well as members whose synthesis is induced by heat stress and by a variety of other adverse stimuli (Parsell and Lindquist, 1994; Voellmy, 1994; Welch, 1992; Linquist and Craig, 1988). In the liver of naive mice, for example, hsp90, hsc70, and hsp60 are expressed constitutively at appreciable levels. Levels of hsp70i are relatively low, and hsp25 is virtually undetectable (Wilkinson and Pollard, 1993; Klemenz et al., 1993; Bardella et al., 1987). In animals subjected to heat stress, levels of hsp70i and hsp25 are increased dramatically (Lu and Das, 1993; Blake et al, 1990).

A number of observations suggest that stress induction of hsp synthesis is mediated by the presence of non-native proteins. For example, overexpression of mammalian proteins unable to fold properly causes activation of hsp genes in bacteria (Goff and Goldberg, 1985), and the injection of chemically-denatured proteins into vertebrate cells increases stress protein synthesis, while injection of the corresponding native protein does not (Ananthan et al., 1986). Proteins of the hsp70 family are known to be capable of binding non-native proteins (Palleros et al., 1991; Hightower et al., 1994) and are thought to chaperone their refolding or elimination. During this binding, the heat shock transcription factor normally bound to hsp70 is released, whereupon it rapidly

oligomerizes to become transcriptionally active (Baler et al., 1992; Abravaya et al., 1992; Baler et al., 1993).

Many hepatotoxicants are bioactivated to reactive intermediates that bind cellular macromolecules including proteins (Plaa, 1993; Lindamood, 1991). It is conceivable that adducted proteins resulting from reactive metabolite binding are physiologically equivalent to proteins unfolded as a consequence of exposure to classical inducers of the heat stress response (e.g., heat, heavy metals, etc.), thereby activating hsp gene expression. Limited studies have suggested that hepatotoxicants forming reactive metabolites may increase the expression of hsps (Salminen et al., 1996), but there has been little direct examination of the relationship between hepatic reactive metabolite formation and hsp gene activation.

This report describes the results of a study of hsp induction in mice treated with hepatotoxic doses of cocaine. Cocaine, like a number of other hepatotoxicants, is metabolized to a reactive metabolite which binds to proteins (Evans, 1983). The role of adducted proteins in the cytotoxicity of cocaine in the liver is unclear, but previous studies have shown that reactive metabolite binding occurs only in the region of the lobule where necrosis subsequently develops — zone 2 in naive ICR mice (Roth et al., 1992). Unlike other classical hepatotoxicants, the location of reactive metabolite binding and toxicity within the hepatic lobule can be altered by pretreatment with hepatic enzyme inducing agents. In mice pretreated with phenobarbital (PB), protein binding and necrosis occur in zone 1, while in b-naphthoflavone (bNF)-pretreated mice they are localized primarily in zone 3 (Roth et al., 1992). In this study, we show a strong correlation between the intralobular location of cocaine reactive metabolite binding and the location of hsp accumulation, suggesting that cocaine reactive metabolite binding to protein plays a role in triggering hsp induction.

## Materials and Methods

Animals and treatments. Adult ICR male mice (Harlan Sprague-Dawley, Indianapolis, IN) weighing 25-30 g were used for these studies. Mice were housed on corn cob bedding in temperature- and humidity-controlled animal quarters with a 12-h light/dark cycle and allowed free access to food and water before and during the experiments. The following pretreatment regimens were used to induce or inhibit cytochrome P-450 activity in the liver: sodium phenobarbital, 80 mg/kg i.p., daily for four days; b-naphthoflavone, 40 mg/kg i.p., once daily for three days; or SKF-525A, 50 mg/kg i.p., 30 minutes before treatment with cocaine. After pretreatment, mice were administered a single i.p. dose of cocaine HCl (Sigma Chemical Co., St. Louis, MO) in saline. All pretreatment and cocaine doses were given with an injection volume of 10 ml/kg body weight. Mice were killed by carbon dioxide asphyxiation. Before initiation of the study, all procedures were assessed and approved by the Institutional Animal Care and Use Committee.

Polyacrylamide gel electrophoresis. Four hundred milligrams of liver was homogenized in 5 ml of sample buffer (0.05 M Tris[hydroxymethyl] aminomethane (Tris), 2% sodium dodecyl sulfate, 10 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, pH 6.8). Each sample was boiled for five minutes, passed through a 22 ga. needle three times to shear DNA, and stored at -80°C until use. Two hundred micrograms of protein from each sample was aliquoted to separate tubes and bromphenol blue was added to a final concentration of 0.0025%. Each aliquot was boiled for five minutes, loaded onto separate lanes of a 12.5% SDS-PAGE gel, and resolved by electrophoresis (Laemmli, 1970).

Protein blotting and immunostaining. Proteins separated by SDS-PAGE were immediately blotted to Hybond-ECL Western membrane (Amersham, England) using a semi-dry blotting apparatus (Millipore, Bedford, MA) and one-half strength Towbin

buffer (10% methanol, 96 mM glycine, and 10 mM Tris-base). Blotting was done at 320 mA for 1.5 hours. Upon completion, the membrane was blocked in TBS (20 mM Tris, 500 mM sodium chloride, pH 7.5) containing 3% gelatin. The membrane was washed two times for five minutes each in TTBS (TBS containing 0.05% polyoxyethylenesorbitan monolaurate) and probed with one of the following antibodies: anti-hsp25 (rabbit polyclonal), anti-hsp60 (mouse monoclonal), anti-hsc70 (mouse monoclonal), anti-hsp70i (mouse monoclonal), or anti-hsp90 (mouse monoclonal). Each of these antibodies were obtained from Stressgen (Victoria, B.C., Canada) and used at a 1:1000 dilution in TTBS containing 1% gelatin. Incubation was for 18 hours at 24°C with continuous shaking. The membrane was washed two times for five minutes each with TTBS and then a sheep anti-mouse or donkey anti-rabbit (depending upon the primary antibody used) horseradish peroxidase-conjugated antibody (Amersham, England) at a 1:3000 dilution in TTBS containing 1% gelatin was incubated with the membrane for one hour at 24°C with continuous shaking. Next, the membrane was washed three times for five minutes each in TTBS and once for five minutes in TBS. The chemiluminescent horseradish peroxidase substrate Luminol (Amersham, England) was added to the membrane and the membrane exposed to standard X-ray film to localize antibody binding.

Immunohistochemical detection of hsp25, hsp70i, and cocaine adducts in murine liver. Five millimeter thick sections from several lobes of each liver were placed in tissue cassettes and fixed in neutral buffered formalin for three hours. The livers were rinsed and stored in saline, processed routinely, and embedded in paraffin. Four sequential sections, 4-6 μm thick, were cut from the same block to facilitate, to the extent possible, cell-by-cell comparisons of cocaine reactive metabolite binding, histopathology, and induced accumulation of hsp25 and hsp70i. One section was stained with hematoxylin and eosin and examined for histopathology by light microscopy, while the remaining sections were immunohistochemically stained with an anti-hsp25 antibody, an anti-hsp70i monoclonal antibody, or an anti-cocaine antibody as follows. Preliminary experiments were

performed to ensure that an adequate blocking reagent was used that would prevent nonspecific binding of immunoglobulins to necrotic areas. TBS containing 25% (v/v) bovine serum plus 3% (w/v) purified bovine serum albumin (BSA) (blocking solution) was found to prevent the non-specific binding to necrotic areas of normal rabbit serum, mouse IgG, or normal sheep serum used as negative controls for the anti-hsp25, anti-hsp70i, or anti-cocaine antibodies, respectively. Fab fragment goat anti-mouse IgG (H+L) (Jackson Immuno Research Laboratories, Inc., West Grove, Pennsylvania) was added to the blocking solution (10 μg/ml final concentration) before blocking slides subsequently probed for hsp70i induction. The latter addition blocked any endogenous mouse IgG that was present in the sections and prevented false positive signals when probing with the biotinylated anti-mouse IgG secondary antibody. Secondary antibody-only treated slides exhibited no binding.

The following procedure was used for immunostaining the sections. Paraffin embedded sections were deparaffinized by passing through three changes of xylene for five minutes each. The sections were passed through 100% ethanol two times for one minute each, 95% ethanol for one minute, and double distilled water ($ddH_2O$) two times for two minutes each. Endogenous peroxidase activity was quenched by submerging the slides in 3% hydrogen peroxide containing 0.1% sodium azide for ten minutes. The slides were then washed in $ddH_2O$ three times for two minutes each and then equilibrated in TBS for at least two minutes. All the following incubations were performed in a humidified chamber. Blocking solution was placed on each section and incubated at 37°C for one hour. The slides were washed two times in TBS for two minutes each. Anti-hsp25 antibody (rabbit polyclonal, Stressgen, B.C., Canada) was diluted 1:100 in blocking solution and placed on the appropriate slides, while anti-hsp70i (mouse monoclonal, Stressgen, B.C., Canada) was diluted 1:100 in blocking solution devoid of the Fab fragment goat anti-mouse IgG antibody and placed on the appropriate slides. Anti-cocaine antibody (sheep polyclonal, JEM Research Products, Inc., Arlington,

Virginia) was diluted 1:100 in blocking solution and placed on the appropriate slides. The antibodies were incubated with the sections at 37°C for one hour and then at 24°C for 18 hours. The sections were washed three times in TBS for two minutes each. Biotinylated goat anti-mouse, goat anti-rabbit, or rabbit anti-sheep (Southern Biotechnology Associates, Inc., Birmingham, AL), depending upon the primary antibody used, was diluted 1:500 in TBS containing 3% BSA, placed on the slides and incubated at 37°C for thirty minutes. The sections were washed three times in TBS for two minutes each. Streptavidin-linked horseradish peroxidase (Southern Biotechnology Associates, Inc., Birmingham, AL) was diluted 1:200 in TBS containing 3% BSA, placed on the slides and incubated at 37°C for thirty minutes. The sections were washed three times in TBS for two minutes each. The horseradish peroxidase colorimetric substrate 3,3'-diaminobenzidine (DAB) (Sigma Chemical Co., St. Louis, MO) supplemented with 0.03% $NiCl_2$ (w/v) was incubated with each section for fifteen minutes at 24°C to provide a permanent location of antibody binding. The sections were then counterstained with hematoxylin and dehydrated by passing through graded alcohols and xylene in the reverse order as for deparaffinizing the sections. The sections were mounted using Permount (Fisher Scientific, Orlando, FL) and a glass cover slip. As a negative control, no binding of the anti-cocaine antibody, using the above procedure, was observed in livers from mice administered necrogenic doses of carbon tetrachloride, bromobenzene, or acetaminophen.

Protein determination. Protein was measured with the Micro Protein Determination assay (Sigma Chemical Co., St. Louis, MO) using BSA as standard.

Serum alanine aminotransferase (ALT) activity. Blood for measurement of serum ALT activity was collected by cardiac puncture immediately after carbon dioxide asphyxiation. Serum ALT activity was determined according to the method of Bergmeyer et al. (1978) using a commercially available kit (Sigma Diagnostics, St. Louis, MO).

Statistical analysis. Data were analyzed by a one-way ANOVA followed by a Student Neuman-Keuls post-hoc test. The level of significant difference was defined as the 0.05 level of probability.

## Results

Initial experiments used Western blot analysis to identify specific hsps induced in response to an hepatotoxic dose of cocaine. Representative Western blots showing the hepatic levels of hsp25, hsp60, hsc70, hsp70i, and hsp90 at 0 (control), 3, 6, and 24 hours after treatment of naive, male ICR mice with cocaine, 50 mg/kg, i.p., are shown in Figure 3-1. The levels of hsp60, hsc70, and hsp90 in the liver were unaffected by cocaine treatment at any of these time points. Levels of hsp25, however, were elevated at 6 and 24 hours, and levels of hsp70i were higher than those in controls at each of the observation times. Concentrations of hsp25 and hsp70i were both greatest at 24 hours.

In a subsequent set of experiments, livers were removed for immunostaining and histopathologic evaluation 0, 3, 6, or 24 hours after a cocaine dose (50 mg/kg, i.p.). Based on the results of the Western blot experiments (described above), immunostaining for hsps was restricted to hsp25 and hsp70i. Following cocaine treatment, swelling of midzonal (zone 2) and centrilobular (zone 3) hepatocytes was observed at the earliest time point — 3 hours — and midzonal necrosis was evident by 6 hours after the cocaine dose (not shown). At 24 hours, the midzonal necrosis was extensive. Immunoreactive cocaine bound to protein was present beginning with the 3 hour specimens and was restricted to cells with altered morphology (i.e., cell swelling). Positive immunostaining for hsp25 and hsp70i was observed 3 hours after the cocaine dose. Hsp25 and hsp70i levels increased over time with the greatest accumulation observed 24 hours post-administration based on the intensity of immunostaining. As with the intralobular distribution of cocaine reactive metabolite binding, hsp25 and hsp70i expression was limited to cells with altered morphology. The extent of immunostaining appeared to be relatively uniform within the

lesion. An example of the relationship between morphology, reactive metabolite binding, and hsp25 and hsp70i levels is shown in Figure 3-3, which is from a liver specimen taken 24 hours after the cocaine dose. Hsp25 and hsp70i accumulation and cocaine binding (Figures 3-3b, c, & d) were superimposable upon the hepatocytes with altered morphology in the midzonal regions (Figure 3-3a). Liver sections from vehicle-treated control mice displayed normal histology and no detectable immunostaining for cocaine reactive metabolite, hsp25, or hsp70i (Figure 3-2 and not shown).

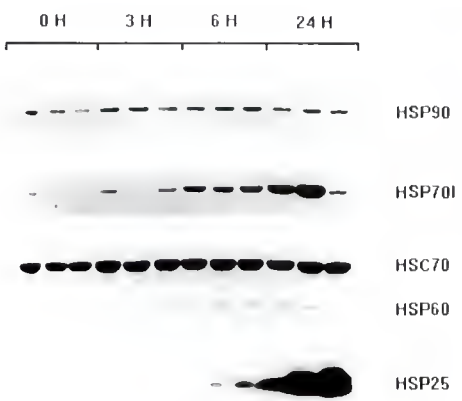



Figure 3-1. Heat shock protein (hsp) levels in murine liver 0, 3, 6, or 24 hours after treatment with 50 mg/kg cocaine. Liver protein was resolved on a 12.5% SDS-PAGE gel, and the relative levels of various hsps estimated by Western blotting using antibodies specific for the indicated hsps. All samples were from the same experiment, with each lane showing hsp levels in a single mouse liver. Equal amounts of protein (i.e., 200 μg) from each sample were loaded onto separate lanes.

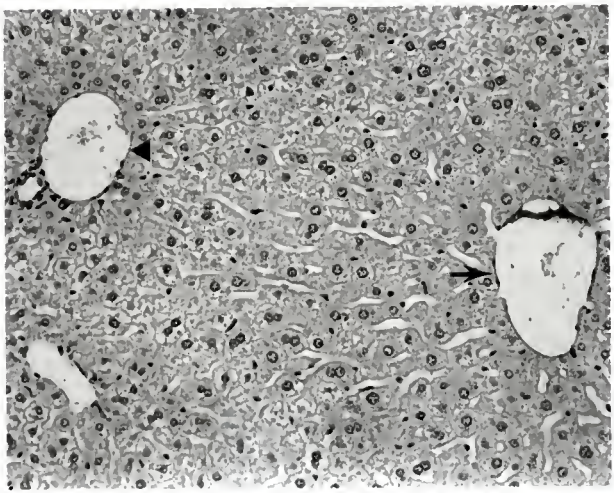
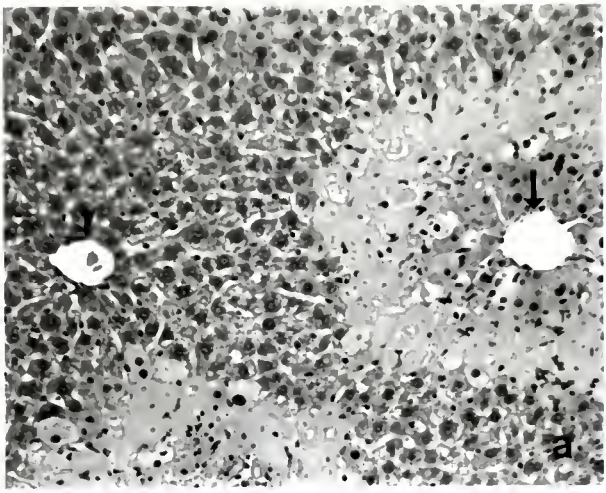


Figure 3-2. Imunohistochemical detection of hsp25 in murine liver 24 hours after treatment of naive mice with saline. The photomicrograph is of an immunohistochemically stained liver section using an anti-hsp25 antibody. The slide was counterstained with hematoxylin. Arrow indicates central vein and arrow head indicates portal triad. Liver histology is unremarkeable and no positive immunostaining is evident. Field magnification 200X.

Figure 3-3. Imunohistochemical detection of hsp25, hsp70i, and cocaine-adducted cellular macromolecules in murine liver 24 hours after treatment of naive mice with 50 mg/kg cocaine. Sequential sections were stained as follows: a) hematoxylin and eosin stain, b) immunohistochemical stain using an anti-hsp25 antibody, c) immunohistochemical stain using an anti-hsp70i antibody, d) immunohistochemical stain using an anti-cocaine antibody. All immunostained slides were counterstained with hematoxylin. Arrow indicates central vein and arrow head indicates portal triad. Midzonal (zone 2) necrosis is evident with some injury occuring closer to the central vein (zone 3). Field magnification 200X.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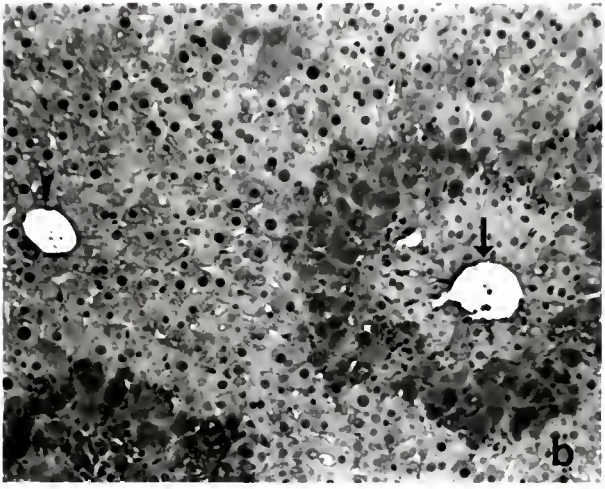
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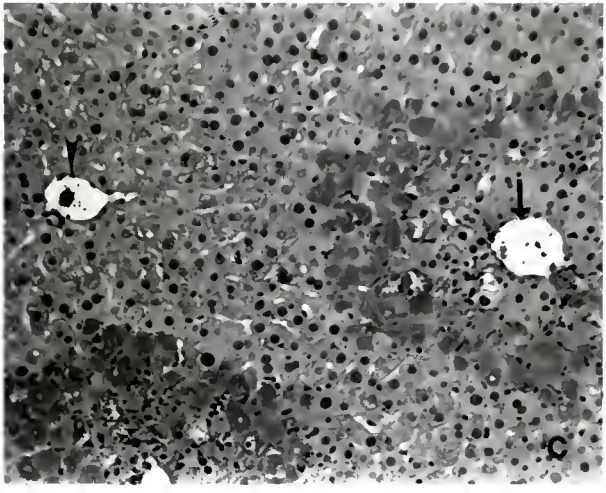
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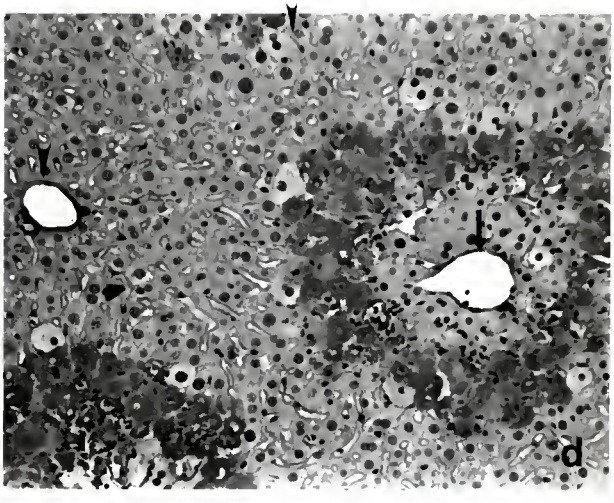
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To test if hsp accumulation following cocaine was a function of reactive metabolite binding and/or hepatotoxicity, rather than a direct effect of cocaine, the ability of the cytochrome P-450 inhibitor SKF 525A to inhibit cocaine binding and cocaine-induced hsp accumulation was measured through immunostaining. Consistent with previous observations (Roth et al, 1992), SKF 525A pretreated animals had little or no hepatic necrosis from cocaine, and the increase in serum alanine aminotransferase (ALT) activity associated with cocaine treatment, indicative of hepatic damage, was abolished (Table 3-1). Also lost in SKF 525A pretreated mice was any detectable immunostaining for cocaine reactive metabolite, hsp25, or hsp70i (not shown).

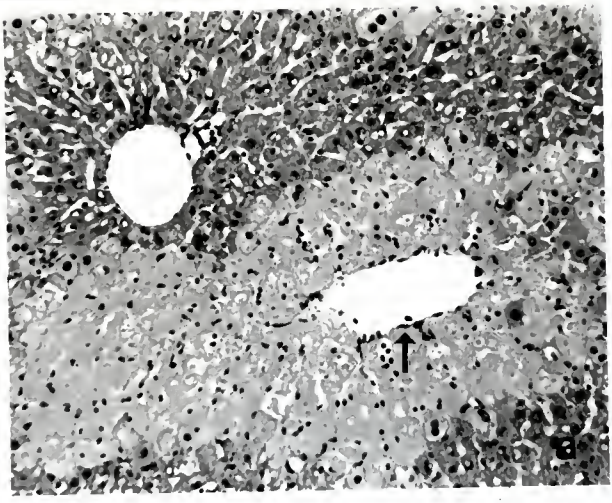
Table 3-1. Effect of SKF-525A pretreatment on serum ALT activities after cocaine administration.

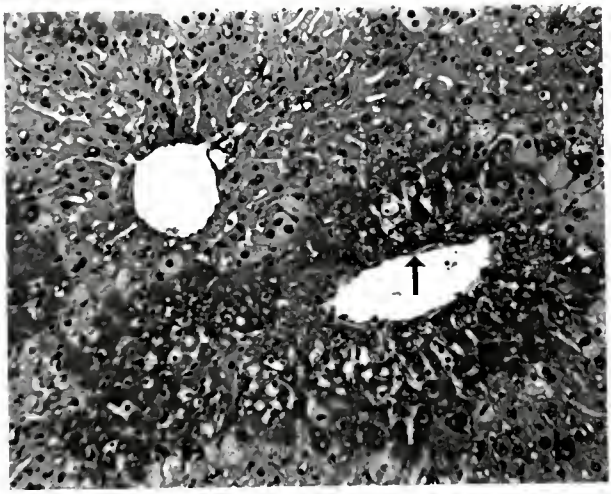
| Treatment | Serum ALT Activity (IU/L) |
|---|---|
| Saline | 54.4 ± 13.3 |
| SKF 525A alone | 58.0 ± 6.2 |
| Cocaine alone | 917.5 ± 410.3 * |
| SKF 525A then Cocaine | 21.8 ± 9.8 |

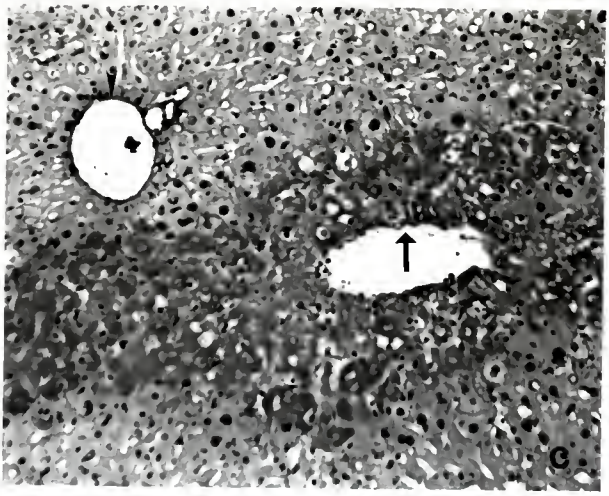
Note. SKF-525A (50 mg/kg, i.p.) was administered 30 minutes prior to administration of cocaine (50 mg/kg, i.p.). Serum ALT activities were measured 24 hours after administration of cocaine. Data expressed as mean ± SEM (n = 5 animals per treatment group). * indicates significantly different from saline treated mice ($p<0.05$).

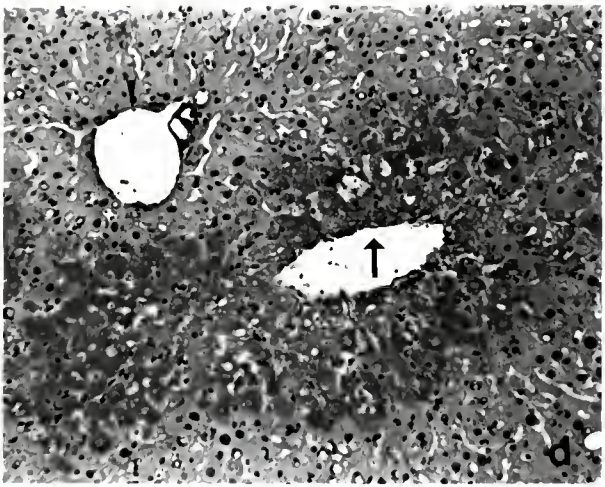
In an additional experiment, mice were pretreated with PB or ßNF to determine if the association between cocaine reactive metabolite binding and hsp accumulation would be retained if the site of the cocaine-induced lesion was shifted. As with naive mice, preliminary experiments using Western blotting confirmed that among the hsps tested, only hsp25 and hsp70i were strongly induced by cocaine in either PB- or ßNF-pretreated

Figure 3-4. Immunohistochemical detection of hsp25 or hsp70i induction and cocaine adduction of cellular macromolecules in murine liver 24 hours after treatment of ß-naphthoflavone-pretreated mice with 50 mg/kg cocaine. Sequential sections were stained as follows: a) hematoxylin and eosin stain, b) immunohistochemical stain using an anti-hsp25 antibody, c) immunohistochemical stain using an anti-hsp70i antibody, d) immunohistochemical stain using an anti-cocaine antibody. All immunostained slides were counterstained with hematoxylin. Arrow indicates central vein and arrow head indicates portal triad. Extensive centrilobular (zone 3) necrosis is evident. Field magnification 200X.









mice (not shown). Consistent with previous observations (Roth et al., 1992), ßNF pretreatment caused an apparent shift in the intralobular site of necrosis, as well as reactive metabolite binding, to the centrilobular region (zone 3) (Figure 3-4). This was accompanied by an identical shift in the intralobular site of accumulation of hsp25 and hsp70i. As was the case with mice without pretreatment, hsp accumulation occurred only in cells with detectable reactive metabolite binding and altered morphology. Mice pretreated with PB, in contrast, displayed hepatic necrosis and reactive metabolite binding predominantly in the peripheral lobular region (zone 1) (not shown). Again, the intralobular sites of accumulation of hsp25 and hsp70i corresponded precisely with the localization of the reactive metabolite binding and lesion.

## Discussion

Heat stress, the prototypical stimulus for increased hsp synthesis, characteristically induces the synthesis of an array of hsps. A survey of the response of the most common hsp families from 25- to 90-kDa to a series of hepatotoxicants in mice found that only hsp25 and hsp70i were increased significantly (Voellmy et al., 1994). The results observed here with cocaine are consistent with this earlier report. The variability observed in the magnitude of hsp induction among mice at a given time point (see Figure 3-1) has been observed previously for other toxicants. Since the degree of toxicity varies also among individual animals within a given treatment group, this is perhaps not surprising.

Hsp70i induction has been observed in rats treated with halothane (VanDyke et al., 1992). Effects of this hepatotoxicant on other hsps is unknown, since this study focused specifically on hsp70i. Goering et al. (1993) incubated liver slices from cadmium-treated rats with $^{35}$S-methionine and observed increased de novo synthesis of proteins with relative molecular masses of 70-kDa, 90-kDa, and 110-kDa. Western blots probed with specific antibodies revealed the 70-kDa protein to be hsp70i (termed hsp72 in

their report) and the 90-kDa protein to be grp94. We have found no increase in grp94 levels following cocaine treatment in mice (unpublished observations), an apparent difference in response that could be related to differences in either hepatotoxicant action between cocaine and cadmium, or to fundamental species differences in response to hepatotoxicants. It is unclear whether there was also a difference in hsp25 response. Goering and colleagues did not observe increased synthesis of proteins with a molecular mass at or near 25-kDa following cadmium treatment as was seen with cocaine. Induction of hsp25 synthesis could conceivably have been missed using $^{35}$S-methionine labeling in their study, however, since rodent hsp25 lacks methionine (Kim et al., 1983).

Metabolism of many hepatotoxic compounds leads to the formation of reactive metabolites, and binding of these metabolites with proteins has been postulated to play a role in their cytotoxic effects. In general, very little is known about the effects of adduction with these relatively small molecular weight metabolites on the conformation of target proteins. It is plausible, however, that adduction of target proteins could lead to their recognition as non-native, causing activation of hsp genes. Cocaine is among the hepatotoxicants whose oxidative metabolism leads to a reactive metabolite (Evans, 1983), and it has been shown previously that immunohistochemistry can be used to identify the intraacinar sites of cocaine reactive metabolite binding (Roth et al., 1992). In order to explore the relationship between reactive metabolite binding and increased hsp synthesis, this technique was used to compare the localization of cocaine-adducted proteins relative to accumulation of hsp25 and hsp70i.

The spatial correlation within the lobule between cocaine reactive metabolite binding, hsp25 and hsp70i accumulation, and cytotoxicity was found to be remarkably consistent. That is, immunostaining of sequential sections revealed that only cells with detectable cocaine metabolite binding had altered morphology and increased concentrations of hsp25 and hsp70i. This correlation existed whether the site of cocaine metabolite binding was in zone 2 (naive mice), zone 1, (PB-induced mice), or zone 3 (bNF

pretreated mice). Cocaine reactive metabolite formation and hepatotoxicity result from cytochrome P-450 mediated oxidation of cocaine (Boelsterli and Goldlin, 1991), and both can be prevented by pretreatment of mice with the cytochrome P-450 inhibitor SKF 525A (Roth et al., 1992). When reactive metabolite formation and binding was prevented by pretreatment with SKF 525A, no increases in hsp25 or hsp70i concentrations were observed. These observations are consistent with a mechanism in which cocaine-adducted protein provides a stimulus for activation of hsp25 and hsp70i genes. Of course, because reactive metabolite binding is closely correlated with cytotoxicity, the possibility cannot be ruled out that hsp induction occurs in response to some facet of toxicity rather than as a direct consequence of protein adduction. The difficulty in experimentally separating cocaine reactive metabolite binding and toxicity makes this possibility difficult to address.

Another interesting observation arising from this study was the relative uniformity of the immunostaining within the affected areas, both for cocaine reactive metabolite binding and the hsps. To the extent that the intensity of staining is reflective of intracellular concentrations, relatively equivalent levels of hsps were observed in cells within the lesions, up to the margins of the affected areas. Cells immediately adjacent to cells in the affected area had no apparent staining. This suggests that hsp induction in response to cocaine may be an "all or none" phenomenon. The absence of a graded hsp response may be a function of a threshold for cocaine reactive metabolite binding to proteins. This would be consistent with the hypothesis that cocaine reactive metabolite binding to protein triggers hsp induction, and would explain why cells lacking cocaine reactive metabolite binding (i.e. cells surrounding and away from the necrotic lesions) did not exhibit hsp induction. The existence of a threshold for reactive metabolite binding is well established for other hepatotoxicants such as acetaminophen and bromobenzene (Casini et al., 1985; Birge et al., 1990; Roberts et al., 1990), and is related to the capacity of intracellular detoxification mechanisms such as glutathione conjugation. The presence of a similar threshold for cocaine has not been explicitly demonstrated, but the apparent

absence of a graded response among cells for cocaine reactive metabolite binding in this study implies that such a threshold exists.

Increased levels of hsp25 and hsp70i were detectable in cocaine-treated mice at the earliest time point examined, 3 hours post-administration, and the hsp concentrations continued to increase during the 24-hour period following the cocaine dose (Figure 3-1 and data not shown). The apparent continued synthesis of the hsps between 6 and 24 hours post-administration was somewhat surprising, given the severity of toxicity experienced by the cells within the lesion. By the 6-hour time point, a number of the cells within the affected zone were necrotic, and by 24 hours severe coagulation necrosis was evident. Continued increases in hsp levels during this time frame suggest that hepatocytes are capable of sustaining hsp synthesis until very near cell death, at least in response to cocaine intoxication.

In conclusion, the results of this study indicate that cocaine hepatotoxicity results in a strong, but selective induction of hsp accumulation in the mouse. Induction of hsp accumulation, as well as cytotoxicity, is confined to cells with detectable cocaine metabolite binding, suggesting that this binding may be responsible for both phenomena. The role of this induced hsp accumulation in the hepatotoxicity of cocaine and other compounds remains to be determined. On one hand, hsp induction may constitute a cellular defense mechanism to enhance the correct refolding of damaged proteins and/or their targeting for degradation (Salminen et al., 1996; Parsell and Lindquist, 1994; Ovelgonne et al., 1995). On the other hand, the continued synthesis of these proteins may contribute to toxicity by further perturbing metabolism of cells attempting to respond to a toxicant insult (Goering et al., 1993). Delineation of the role of hsps in toxicant action will be an important step in understanding the fundamental mechanisms of hepatotoxicity.

# CHAPTER 4
# DIFFERENTIAL HEAT SHOCK PROTEIN INDUCTION BY ACETAMINOPHEN AND A NON-HEPATOTOXIC REGIOISOMER, 3'-HYDROXYACETANILIDE, IN MOUSE LIVER

## Introduction

Recent studies have shown that levels of certain heat shock proteins (hsps) are increased in the liver in response to hepatotoxicants. The livers of rats treated with halothane have elevated levels of hsp70i (also termed hsp72) (VanDyke et al., 1992), and cadmium treatment in rats results in increased synthesis of hsp70i, grp94 and a 110-kDa protein (Goering et al., 1993). Mice treated with hepatotoxic doses of cocaine have elevated hepatic levels of hsp25 and hsp70i (Salminen et al., 1997). The significance of increased de novo synthesis of these proteins in response to hepatotoxicants is unclear, although hsp induction has been correlated with protection from some hepatotoxicants (Salminen et al., 1996). These observations, when taken together with the fact that hsps function as chaperones (for reviews see Jaattela and Wissing, 1992; Welch, 1992; Lindquist and Craig, 1988), suggest that hsp induction may constitute an important cell defense mechanism against proteotoxic chemicals.

Activation of heat shock transcription factor (HSF), the factor responsible for the activation of hsp genes during stress (Baler et al., 1993; Abravaya et al., 1992), appears to result from the presence within the cell of non-native proteins (Ananthan et al., 1986; Zuo et al., 1995). It is reasonable to postulate that adduction of proteins by reactive metabolites of hepatotoxicants may render them sufficiently "non-native" to enable them to trigger the activation of HSF, resulting in upregulation of hsp synthesis. Indirect evidence is provided by recent studies comparing the intralobular localization of hsp accumulation and reactive metabolite binding from cocaine (Salminen et al., 1997). In mice treated with an

hepatotoxic dose of cocaine, elevated hsp levels were observed only in cells with detectable cocaine reactive metabolite binding, and shifting the location within the lobule of metabolite binding through pretreatment with phenobarbital or ß-naphthoflavone produced a corresponding shift in cells expressing the hsps. However, because reactive metabolite binding was also correlated with cytotoxicity, the possibility could not be ruled out that increased hsp expression occurred in response to some manifestation of toxicity rather than as a direct consequence of protein adduction.

In an effort to discriminate between protein adduction versus some unidentified facet of cytotoxicity as a stimulus for hsp synthesis, additional studies were conducted using acetaminophen (APAP) and its regioisomer 3'-hydroxyacetanilide (AMAP). APAP is a commonly used analgesic/antipyretic agent that can cause liver necrosis in overdose situations (Boyer and Rouff, 1971; Ambre and Alexander, 1977; Hinson, 1980). Cytochrome P-450-mediated oxidation of APAP results in the formation of an electrophilic intermediate, N-acetyl-p-quinone imine (NAPQI), that is detoxified by conjugation with glutathione (GSH) (Plaa, 1993; Corcoran et al., 1980; Dahlin et al., 1984). Following hepatotoxic doses of APAP, hepatocellular GSH becomes depleted, permitting unconjugated NAPQI to bind cellular macromolecules (Manautou et al., 1996; Rashed et al., 1990). Though many possible nucleophilic targets exist in the cell, certain proteins are preferentially bound by NAPQI and their adduction has been proposed to play a role in causing cell death (Bartolone et al., 1992; Bulera et al., 1995).

AMAP also undergoes oxidation to produce electrophilic intermediates which bind to proteins. Based on GSH conjugates identified following AMAP administration to mice, at least three AMAP metabolites (viz., 2-acetamido-p-benzoquinone, 4-acetamido-o-benzoquinone, and N-acetyl-3-methoxy-p-benzoquinone imine) have been postulated to account for this binding (Rashed and Nelson, 1989). While equimolar doses of APAP and AMAP produce similar levels of covalently bound metabolites, AMAP is not hepatotoxic (Roberts et al., 1990). Even when the AMAP dose was increased to its highest non-lethal

amount, and peak reactive metabolite binding was nearly twice that of an hepatotoxic dose of APAP, no liver injury from AMAP was observed, and biochemical effects associated with APAP were absent (i.e., perturbation of calcium homeostasis, inhibition of mitochondrial function, and inhibition of glutathione peroxidase and thioltransferase activity) (Tirmenstein and Nelson, 1989; Rashed et al., 1990; Tirmenstein and Nelson, 1990).

In the study reported here, APAP and AMAP were compared with respect to toxicity, reactive metabolite binding, and effects on hepatic hsp levels. The difference between these two structurally related compounds in terms of hepatotoxic effect was verified. Total hepatic hsp responses were evaluated using SDS-PAGE and Western blots, and potential differences in responses among cells within the hepatic lobule were examined using immunohistochemistry. Also, the distribution of cells expressing hsps was compared with the intralobular distribution of reactive metabolite binding.

## Materials and Methods

Animals and treatments. Adult B6C3F1 male mice (Harlan Sprague-Dawley, Indianapolis, IN) weighing 22-25 g were used. Mice were housed on corn cob bedding in temperature- and humidity-controlled animal quarters with a 12-h light/dark cycle, and allowed free access to water before and during the experiments. Mice were fasted for 16 hours before APAP (4'-hydroxyacetanilide) or AMAP (3'-hydroxyacetanilide) (Sigma Chemical Co., St. Louis, MO) doses and then given food for the duration of the experiments. Mice were administered a single i.p. dose of 200 mg/kg APAP or 1000 mg/kg AMAP in warm saline. The dose for each compound was determined in preliminary experiments to be the maximum tolerated dose that allowed survival for the duration of the experiments. Some mice were pretreated one hour prior to the AMAP dose with L-butathionine-[S,R]-sulfoximine (BSO; 222 mg/kg, i.p.) dissolved in saline. APAP and BSO were given with an injection volume of 10 ml/kg body weight. AMAP was given with

an injection volume of 20 ml/kg since its limited solubility precluded using a smaller injection volume. Mice were killed by carbon dioxide asphyxiation.

Polyacrylamide gel electrophoresis, protein blotting, and immunostaining. Hsp levels in total liver protein were detected by SDS-PAGE and Western blotting as described previously (Salminen et al., 1996b) with the following modifications. Four hundred milligrams of liver was homogenized in sample buffer (0.05 M Tris[hydroxymethyl]aminomethane (Tris), 2% sodium dodecyl sulfate, 10 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, pH 6.8), boiled for five minutes, passed through a 22 ga. needle three times to shear DNA, and stored at -80°C until use. Two hundred micrograms of protein from each sample was aliquoted to separate tubes and bromphenol blue added to a final concentration of 0.0025%. Each aliquot was boiled for five minutes, loaded onto separate lanes of a 12.5% SDS-PAGE gel, and resolved by electrophoresis. Proteins separated by SDS-PAGE were immediately blotted to Hybond-ECL Western membrane (Amersham, England). Upon completion, the membrane was blocked in TBS (20 mM Tris, 500 mM sodium chloride, pH 7.5) containing 3% gelatin and then probed with one of the following antibodies: anti-hsp25 (rabbit polyclonal), anti-hsp60 (mouse monoclonal), anti-hsc70 (mouse monoclonal), anti-hsp70i (mouse monoclonal), or anti-hsp90 (mouse monoclonal). Each of these antibodies were obtained from Stressgen (Victoria, B.C., Canada) and used at a 1:1000 dilution in TTBS (TBS containing 0.05% polyoxyethylenesorbitan monolaurate) containing 1% gelatin. Incubation was for 18 hours at 24°C with continuous shaking. Primary antibody binding was detected using a sheep anti-mouse or donkey anti-rabbit (depending upon the primary antibody used) horseradish peroxidase-conjugated antibody (Amersham, England) at a 1:3000 dilution in TTBS containing 1% gelatin. The chemiluminescent horseradish peroxidase substrate Luminol (Amersham, England) was added to the membrane and the membrane exposed to standard X-ray film to localize antibody binding.

Immunohistochemical detection of hsp25, hsp70i, and APAP adducts in mouse liver. Five millimeter thick sections from several lobes of each liver were placed in tissue cassettes and fixed in neutral buffered formalin for three hours. The livers were rinsed and stored in saline, processed routinely, and embedded in paraffin. Four sequential sections 4-6 µm thick were cut from the same block to facilitate comparison of localization of hsp induction, APAP or AMAP reactive metabolite binding, and morphologic changes. One section was stained with hematoxylin and eosin and examined for histopathology by light microscopy, while the remaining sections were immunohistochemically stained with an anti-hsp25 antibody, an anti-hsp70i monoclonal antibody, or an anti-APAP antibody as follows. Paraffin embedded sections were deparaffinized by passing through three changes of xylene for five minutes each. The sections were passed through 100% ethanol two times for one minute each, 95% ethanol for one minute, and double distilled water (dd$H_2O$) two times for two minutes each. Endogenous peroxidase activity was quenched by submerging the slides in 3% hydrogen peroxide containing 0.1% sodium azide for ten minutes. The slides were then washed in dd$H_2O$ three times for two minutes each and then equilibrated in TBS for at least two minutes. All the following incubations were performed in a humidified chamber. Blocking solution (TBS containing 25% v/v bovine serum plus 3% w/v purified bovine serum albumin) was placed on each section and incubated at 37°C for one hour. Fab fragment goat anti-mouse lgG (H+L) (Jackson Immuno Research Laboratories, Inc., West Grove, Pennsylvania) was added to the blocking solution (10 µg/ml final concentration) before blocking slides subsequently probed for hsp70i induction. The latter addition blocked any endogenous mouse IgG that was present in the sections and prevented false positive signals when probing with the biotinylated anti-mouse lgG secondary antibody. The slides were washed two times in TBS for two minutes each. Anti-hsp25 antibody (rabbit polyclonal, Stressgen, B.C., Canada) was diluted 1:100 in blocking solution and placed on the appropriate slides, while anti-hsp70i (mouse monoclonal, Stressgen, B.C., Canada) was diluted 1:100 in blocking solution devoid of

the Fab fragment goat anti-mouse IgG antibody and placed on the appropriate slides. Anti-acetaminophen antibody was diluted 1:100 in blocking solution and placed on the appropriate slides. The antibodies were incubated with the sections at 37°C for one hour and then at 24°C for 18 hours. The sections were washed three times in TBS for two minutes each. Biotinylated goat anti-mouse or goat anti-rabbit (Southern Biotechnology Associates, Inc., Birmingham, AL), depending upon the primary antibody used, was diluted 1:500 in TBS containing 3% BSA, placed on the slides, and incubated at 37°C for thirty minutes. The sections were washed three times in TBS for two minutes each. Streptavidin-linked horseradish peroxidase (Southern Biotechnology Associates, Inc., Birmingham, AL) was diluted 1:200 in TBS containing 3% BSA, placed on the slides and incubated at 37°C for thirty minutes. The sections were washed three times in TBS for two minutes each. The horseradish peroxidase colorimetric substrate 3,3'-diaminobenzidine (DAB) (Sigma Chemical Co., St. Louis, MO) supplemented with 0.03% $NiCl_2$ (w/v) was incubated with each section for fifteen minutes at 24°C to provide a permanent location of antibody binding. The sections were then counterstained with hematoxylin and dehydrated by passing through graded alcohols and xylene in the reverse order as for deparaffinizing the sections. The sections were mounted using Permount (Fisher Scientific, Orlando, FL) and a glass cover slip. Using this procedure, no binding of normal rabbit serum or mouse IgG, used as negative controls for the anti-hsp25 and anti-APAP, or anti-hsp70i antibodies, respectively, was observed. In addition, secondary antibody-only treated slides exhibited no binding.

The anti-APAP antibody used in this study has been characterized extensively in two past reports (Matthews et al., 1996; Matthews et al., 1997). It was raised against a protein conjugate of the arylacetamide, N-acetyl-p-aminobenzoic acid, and has a high affinity and specificity for the acetamide and ring portions of the APAP molecule. Due to this specificity, it can recognize the parent compound (APAP), NAPQI, AMAP, and reactive metabolites formed from AMAP that retain the arylacetamide portions of the parent

molecule. We have shown that cytochrome P-450 inhibitors can prevent the immunostaining in liver from mice treated with AMAP or APAP indicating that the detected binding is due to covalently bound metabolites and not due to unbound parent compound (unpublished observations). The anti-APAP antibody exhibited no binding in livers from mice given an acute necrogenic dose of carbon tetrachloride, bromobenzene, or cocaine indicating that antibody binding could not be attributed to unmasking of an endogenous antigen during necrosis. Further, preincubation of the anti-APAP antibody with 1 mM APAP at 37°C for one hour before its addition to the slides prevented the binding of the antibody to the livers from APAP or AMAP treated mice. This observation provided additional confirmation that the antibody specifically recognized protein-bound APAP and AMAP metabolites.

Liver non-protein sulfhydryl (NPSH) depletion. Liver NPSH levels were measured as the total acid soluble thiols according to the method of Ellman (1959). Each liver was homogenized in 5 ml of 6% trichloroacetic acid (w/v) and 1 mM [ethylenedinitrilo]tetraacetic acid and then centrifuged at 1000 x g at 4°C for fifteen minutes. Eighty microliters of supernatant solution was added to 2 ml of phosphate buffer (0.1M, pH 8.0). After addition of 40 µl of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; 4 mg/ml in 95% ethanol), the solution was vortex mixed and allowed to stand at 24°C for five minutes. The absorbance at 412 nm was then measured and the corresponding NPSH concentrations determined using an extinction coefficient of 13,100 $M^{-1}$ $cm^{-1}$.

Protein determination. Protein was measured with the Micro Protein Determination assay (Sigma Chemical Co., St. Louis, MO) using BSA as standard.

Statistical analysis. NPSH data were analyzed by a one-way ANOVA followed by a Student Neuman-Keuls post-hoc test. The level of significant difference was defined as the 0.05 level of probability.

## Results

Western blot analysis was used to measure the induction of hsp accumulation in mouse liver by APAP or AMAP. Representative Western blots in Figure 4-1 show the hepatic levels of hsp25, hsp60, hsc70, hsp70i, and hsp90 at 0 (control), 3, 6, and 24 hours after treatment of naive, male B6C3F1 mice with APAP, 200 mg/kg, i.p. The levels of hsp60, hsc70, and hsp90 in the liver were unaffected by APAP treatment, while hsp25 levels were increased at 6 and 24 hours and hsp70i levels were increased at each time point. Maximal accumulation was observed at 24 hours for both hsps. No increase in any of the hsps was observed in response to AMAP, 1000 mg/kg, i.p. (not shown).

Using information gained from Western blots identifying the hsps increased by APAP treatment, subsequent experiments utilized immunohistochemistry to determine the distribution of hsp25 and hsp70i accumulation within the lobule. In addition, the intralobular location of covalently bound APAP or AMAP was determined immunohistochemically using an anti-APAP antibody. Visible increases in hsp25 and hsp70i immunostaining were observed at the earliest observation time, 3 hours after the dose (not shown). Consistent with the Western blot experiments, hsp25 and hsp70i levels were greatly increased at 6 and 24 hours. Hsp25 and hsp70i accumulation at 6 hours was evident throughout the centrilobular region, and the distribution of cells with increased hsps, covalently bound APAP, and mild morphological changes (i.e. cell swelling) were essentially superimposable (not shown). By 24 hours, the pattern of hsp25 induction had changed dramatically. At that time, hsp70i accumulation was still uniform throughout the centrilobular region and restricted to necrotic hepatocytes (Figure 4-2). In contrast, hsp25 accumulation was minimal within the most affected centrilobular hepatocytes and strongest

in hepatocytes on the periphery and surrounding the lesions. Similar to observations at 6 hours, hsp25 and hsp70i accumulation was restricted to hepatocytes that had covalently-bound APAP.

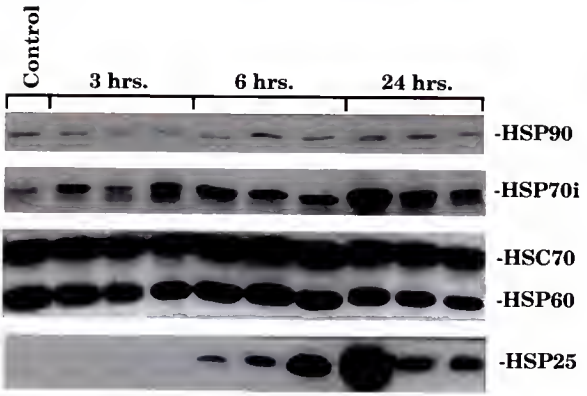
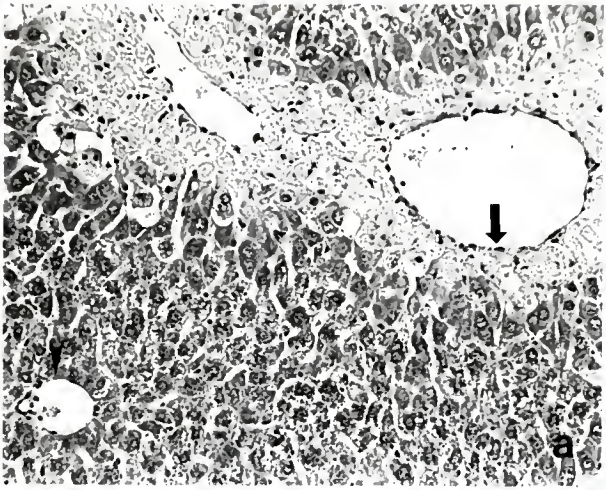



Figure 4-1. Heat shock protein (hsp) induction in mouse liver 0, 3, 6, or 24 hours after treatment with 200 mg/kg APAP. Liver protein was resolved on a 12.5% SDS-PAGE gel, and the level of various hsps determined by Western blotting using an antibody specific for the indicated hsp. The Western blots are from a single experiment with each lane containing a liver sample from a single mouse. Equal amounts of protein (i.e., 200 μg) from each sample was loaded onto separate lanes.

In contrast to the hepatocellular swelling and necrosis produced by APAP, no significant morphological changes were observed in mice treated with AMAP, 1,000 mg/kg. i.p., up to 24 hours after the dose (Figure 4-3). To confirm reactive metabolite binding from AMAP, liver sections from AMAP-treated mice were immunostained. As has been observed elsewhere (Salminen et al., 1997- submitted for publication), the pattern of binding was panlobular rather than localized in the centrilobular region as is the case with APAP. As reflected by the intensity of immunostaining, AMAP binding was greatest one hour after the dose and somewhat reduced at 3 and 6 hours post-treatment. AMAP binding was barely detectable 24 hours after the dose. It is possible that Western blotting (as shown for APAP in Figure 4-1) could have been insufficiently sensitive to detect overexpression of hsps in a small subset of cells. For this reason, liver sections from AMAP-treated mice were also immunostained for hsp25 and hsp70i. No immunostaining for hsp25 or hsp70i was detected anywhere in the lobule at any of the time points (Figure 4-3).

Previous reports that cellular thiol status may be important in hsp gene activation prompted an additional experiment in which the effect of APAP and AMAP on hepatic non-protein sulfhydryls (NPSH) was measured. APAP caused a rapid decline in NPSH to 21% of control values by one hour post-administration (Figure 4-4). NPSH levels began rising by 3 hours and exceeded control levels at 24 hours. The depression in hepatic

Figure 4-2. Immunohistochemical detection of hsp25 and hsp70i accumulation and APAP adduction of cellular macromolecules in mouse liver 24 hours after treatment of naive mice with 200 mg/kg APAP. Four sequential sections were cut to facilitate comparison of hsp induction with APAP adduction and morphological changes. The slides were treated as follows: a) hematoxylin and eosin stain; b) immunohistochemical stain using an anti-hsp25 antibody; c) immunohistochemical stain using an anti-hsp70i antibody; d) immunohistochemical stain using an anti-APAP antibody. All immunostained slides were counterstained with hematoxylin. Arrow indicates portal triad; arrow head indicates central vein.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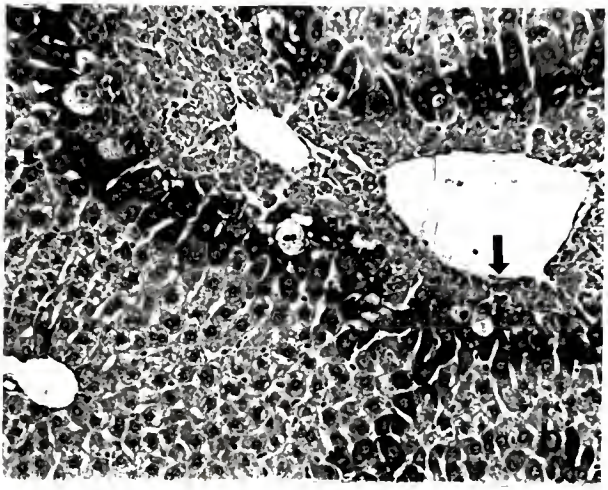
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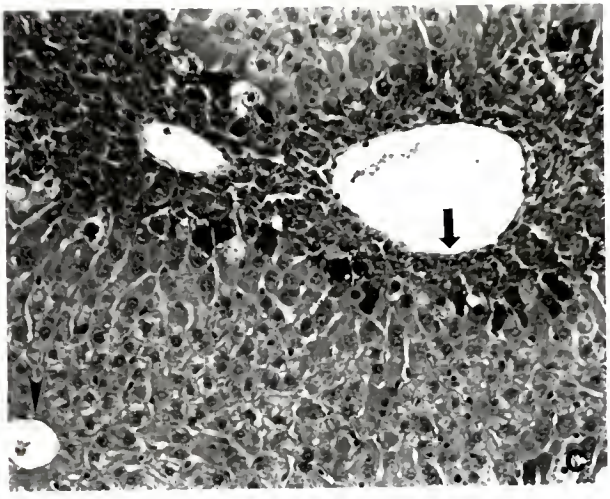


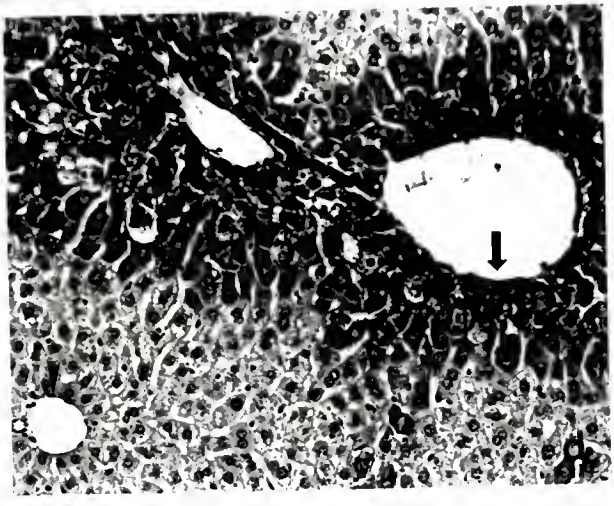
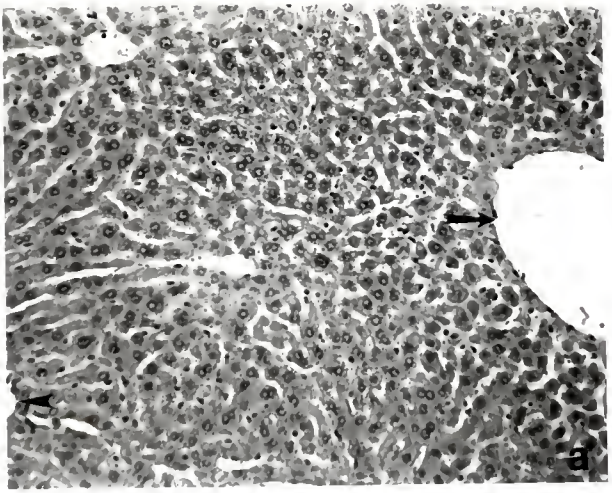


Figure 4-3. Immunohistochemical detection of hsp25 and hsp70i levels and AMAP adduction of cellular macromolecules in mouse liver three hours after treatment of naive mice with 1,000 mg/kg AMAP. Four sequential sections were cut to facilitate comparison of hsp induction with AMAP adduction and morphological changes. The slides were treated as follows: a) hematoxylin and eosin stain; b) immunohistochemical stain using an anti-hsp25 antibody; c) immunohistochemical stain using an anti-APAP antibody. All immunostained slides were counterstained with hematoxylin. Arrow indicates portal triad; arrow head indicates central vein.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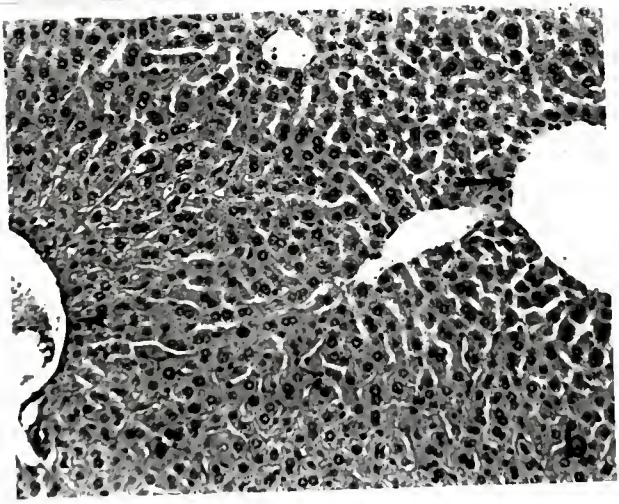
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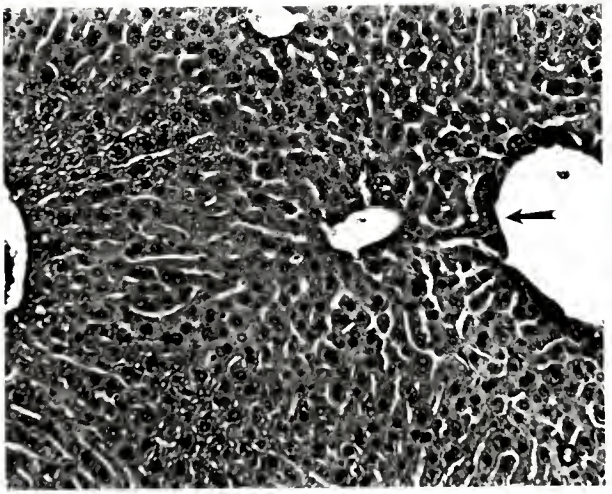


NPSH produced by AMAP was much less pronounced, with a nadir at 46% of control observed 6 hours after the dose. The depression of hepatic NPSH was more protracted than after APAP, however. These data suggested that the lower efficacy of AMAP in depleting NPSH levels might be a possible explanation of its inability to trigger hsp induction.

In order to produce an hepatic NPSH depression in AMAP-treated mice similar to that observed in animals treated with APAP, mice were pretreated with the glutathione synthesis inhibitor butathionine sulfoximine (BSO) one hour prior to the AMAP dose. The dose of AMAP was lowered to 600 mg/kg for these experiments since greater than 50% mortality was observed in mice administered BSO + 1,000 mg/kg AMAP. Despite using a lower AMAP dose, the BSO + AMAP treatment resulted in only a slightly lower level of binding of AMAP to liver protein compared to the 1,000 mg/kg AMAP dose with no change in the pattern of binding (i.e. binding was still panlobular with the greatest binding in the single layer of hepatocytes surrounding the central veins), as measured by immunostaining (not shown). The BSO + AMAP treatment produced a similar decrease in NPSH as APAP at one hour (26% for BSO + AMAP vs. 21% for APAP) with a nadir of 14% at three hours (Figure 4-4). BSO alone caused a maximal decline of NPSH of 52 and 46% of control levels 1 and 3 hours after the dose, respectively (not shown). Similar to AMAP alone, BSO + AMAP failed to cause hepatotoxicity detectable by light microscopy at any of the observation times (Figure 4-5). Western blot analysis of hsp25 and hsp70i induction by BSO + AMAP was inconclusive, but suggested that a slight induction of hsp25 might have occurred by 24 hours (not shown). Immunohistochemical detection of hsp25 accumulation confirmed that BSO + AMAP did produce hsp25 accumulation by 24 hours in a small fraction of cells surrounding the central veins of the liver with no concurrent induction of hsp70i (Figure 4-5). BSO treatment alone failed to cause induction of either hsp as measured by Western blotting and immunohistochemistry (not shown).

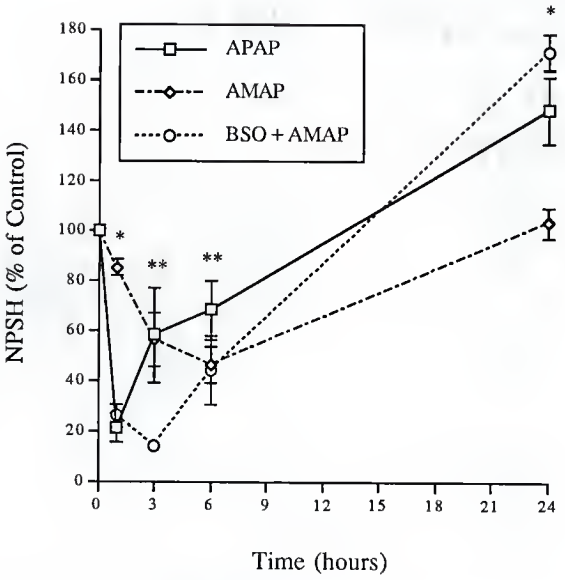
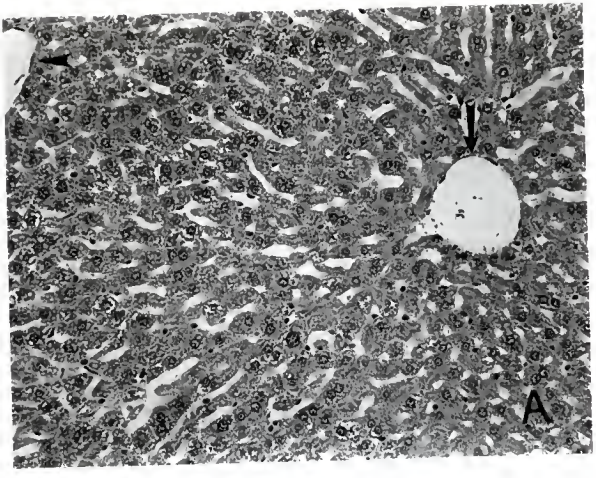



Figure 4-4. The effect of APAP, AMAP, and butathionine sulfoximine (BSO) + AMAP on the liver levels of non-protein sulfhydryls (NPSH). Mice were administered 200 mg/kg APAP or 1,000 mg/kg AMAP and the level of NPSH in the liver measured 0, 1, 3, 6, or 24 hours after the dose as described in Materials and Methods. Some mice were pretreated with BSO (222 mg/kg) one hour prior to the AMAP dose. The AMAP dose was lowered to 600 mg/kg for the combined BSO + AMAP treatment since the 1,000 mg/kg AMAP dose produced an unacceptably high level of mortality. Results are expressed as percentage of the mean NPSH concentration in concurrently euthanized, saline-treated controls. Data are displayed as the mean ± SEM (n = 3 - 4 mice). * denotes a statistically significant decrease/increase in NPSH caused by the APAP or BSO + AMAP treatments compared to control levels; ** denotes a statistically significant decrease in NPSH caused by all three treatments compared to control levels ($p < 0.05$).

Figure 4-5. Immunohistochemical detection of hsp25 and hsp70i accumulation in mouse liver 24 hours after treatment of naive mice with butathionine sulfoximine (BSO) + AMAP. Mice were pretreated with BSO (222 mg/kg) one hour prior to the AMAP dose (600 mg/kg). Three sequential sections were cut to facilitate comparison of hsp induction with morphological changes. The slides were treated as follows: a) hematoxylin and eosin stain; b) immunohistochemical stain using an anti-hsp25 antibody; c) immunohistochemical stain using an anti-hsp70i antibody. All immunostained slides were counterstained with hematoxylin. Arrow indicates portal triad; arrow head indicates central vein.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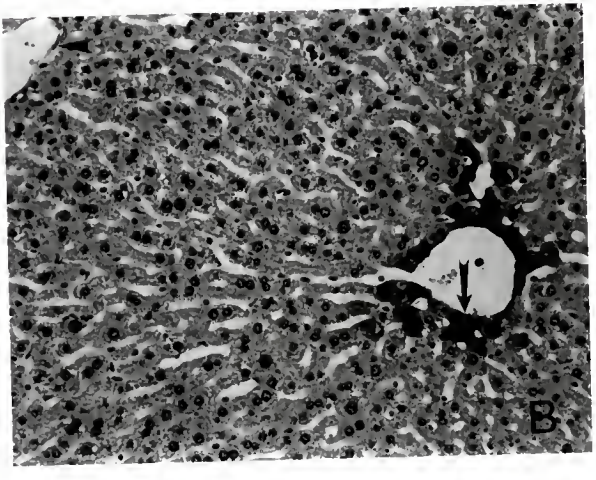
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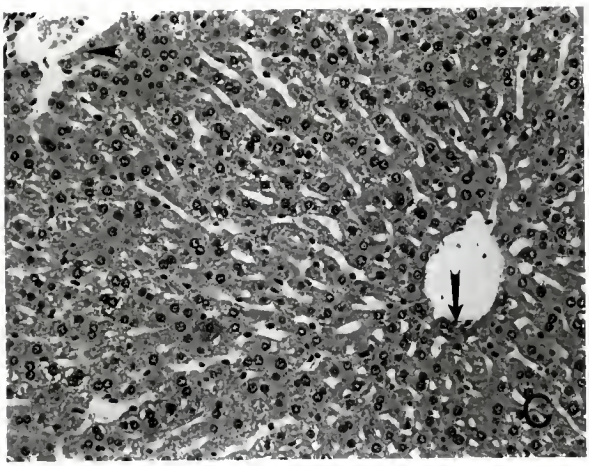
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## Discussion

The results of this study show that toxicity from APAP, like that from a number of other hepatotoxicants, is accompanied by increased hepatic concentrations of hsps. Consistent with previous observations with cocaine, bromobenzene, and carbon tetrachloride (Salminen et al., 1997, Roberts et al., 1996), induction of hsps selectively involved hsp25 and hsp70i — no changes in hsp60, hsc70, and hsp90 were detected. Western blot analysis appeared to suggest that accumulation of hsp70i may precede that of hsp25 (see Figure 4-1). This distinction is probably an artifact resulting from the limited sensitivity of measuring whole tissue hsps by this method. Using immunohistochemical staining, both hsp25 and hsp70i concentrations were increased at the earliest time point examined, i.e., 3 hours after APAP administration. Observations in this study suggest that hsp25 may, in fact, be a more sensitive indicator of cytotoxicity. At 24 hours after the APAP dose, cells at the margin of the lesions had detectable reactive metabolite binding and appeared to be only minimally affected. Within these cells, hsp25 but not hsp70i was elevated (see Figure 4-2). Additional evidence is provided by the observation that combined BSO + AMAP treatment caused induction of only hsp25 (see Figure 4-5).

The strong correlation in intralobular distribution of APAP reactive metabolite binding and increased levels of hsp25 and hsp70i is consistent with protein adduction as a stimulus for hsp synthesis. On the other hand, the absence of increased levels of hsps in response to AMAP, despite extensive reactive metabolite binding, argues that protein adduction alone may not be an effective trigger for hsp upregulation. The latter observation must be interpreted cautiously, as there are a number of potential explanations for the lack of an hsp response. One is that it may not be protein adduction per se, but adduction of certain critical proteins that leads to increased hsp synthesis. There is ample evidence that the reactive metabolites of APAP and AMAP adduct different proteins (Tirmenstein and Nelson, 1989; Myers et al. 1995), and it has been postulated that the greater reactivity of

AMAP metabolites leads to binding with more proximal, and less critical, cellular protein targets than is the case with APAP (Rashed et al., 1990; Ramsay et al., 1989). Differences in hsp induction between APAP and AMAP may reflect differences in the intracellular site(s) of the adducted proteins, or perhaps the nature of the adducted proteins themselves. Tirmenstein et al. (1991) showed that pretreatment with BSO caused an increase in binding of AMAP to mitochondrial proteins. Even though we did not explicitly show increased binding of AMAP to mitochondrial proteins by BSO pretreatment in our study, the combined BSO + AMAP treatment's ability to trigger hsp25 induction might be representative of the increased binding of AMAP to critical cellular targets that were not accessible without BSO pretreatment.

Another possibility relates to the amount or concentration of adducted protein that may be required to trigger increased hsp synthesis. Previous studies have shown the overall extent of binding of AMAP and APAP metabolites to hepatic protein to be similar (Roberts et al., 1990; Rashed et al., 1990). Immunohistochemical staining shows that AMAP reactive metabolite binding occurs in hepatocytes throughout the liver, however, while binding of APAP metabolites is confined to the centrilobular region (see Figures 4-2 and 4-3). The more diffuse binding of AMAP metabolites may result in a lower amount or concentration of adducted protein per cell, perhaps less than the threshold required to trigger hsp synthesis. Increasing adducted protein concentrations by increasing the AMAP dose in order to test this hypothesis is unfortunately precluded by the respiratory depression which occurs at higher dosages of AMAP (Rashed et al., 1990). The ability of BSO + AMAP to trigger hsp25 induction around the central veins is compatible with the notion that the concentration of adducted protein may play a critical role in triggering hsp induction. While AMAP binding occurs panlobularly, the greatest binding occurs in the single layer of hepatocytes surrounding the central veins (see Figure 4-3) which is where hsp25 induction occurs after BSO + AMAP treatment.

Another possible explanation for the absence of hsp induction following AMAP treatment is that protein adduction alone is an insufficient stimulus. Chen et al. (1992) found that binding of an electrophilic metabolite of nephrotoxic cysteine conjugates to protein in LLC-PK1 cells was associated with induction of hsp70 mRNA and increased hsp70 synthesis. Treatment of cells with the thiol-reducing agent dithiothreitol did not affect protein adduction, but nonetheless inhibited induction of hsp70 mRNA. Based on these observations, the authors have proposed that a combination of protein adduction and alterations in cellular non-protein thiols may be needed to activate hsp transcription factor. Huang et al. (1994) found that dithiothreitol prevented induction of hsps by hyperthermia, further suggesting that oxidation of cellular thiols is an important trigger of hsp induction.

In the present study, both APAP and AMAP diminished hepatocellular NPSH content, but the effect of AMAP was relatively modest compared with APAP. When NPSH depletion from AMAP was enhanced by BSO pretreatment to produce declines in NPSH levels comparable to those caused by APAP, accumulation of hsp25 was observed, albeit only in cells surrounding the central vein in which AMAP binding was greatest. These results are consistent with a requirement for both protein adduction and a substantial loss of reduced thiols in triggering hsp25 induction. It is unclear why the hsp25 response in BSO + AMAP treated mice was restricted to cells surrounding the central vein, but there are several possible explanations. As discussed above, the level of protein adduction in other cells may have been below some threshold requirement, the nature of the protein adducts may be different in different regions of the liver, the extent of NPSH depletion in other cells may have been lesser and insufficient to permit the hsp25 response, or some combination of these factors. The absence of an hsp70i response in BSO + AMAP treated animals suggests that there may be somewhat different requirements in terms of cellular events for hsp70i versus hsp25 induction. This hypothesis is supported by the observed differences in localization of hsp25 and hsp70i within the hepatic lobule after hepatotoxic doses of APAP (see Figure 4-2).

In conclusion, hepatotoxic doses of APAP in mice lead to increased levels of hsp25 and hsp70i in the liver. As has been observed previously with cocaine, there is a close temporal and spatial correlation within the lobule of reactive metabolite binding, cytotoxicity, and accumulation of these hsps. The absence of similar increases in hsps in response to AMAP, despite reactive metabolite binding similar in extent to that from APAP (at least on a whole organ basis), implies that the simple presence of adducted protein may not be a sufficient stimulus for increased hsp gene expression. It appears that, at least for hsp25, a reduction in NPSH levels may have to occur simultaneously with protein adduction in order to trigger hsp induction.

# CHAPTER 5
# PROTECTION AGAINST HEPATOTOXICITY BY A SINGLE DOSE OF AMPHETAMINE: THE POTENTIAL ROLE OF HEAT SHOCK PROTEIN INDUCTION.

## Introduction

Amphetamine and methamphetamine are commonly abused drugs that cause peripheral adrenergic and central nervous system stimulation. A common side effect of amphetamine and similar compounds in humans and rodents is hyperthermia, during which core body temperatures often exceed 40°C in overdose situations (Bushnell and Gordon, 1987; Seale et al., 1985; Nowak, 1988; Callaway and Clark, 1994). Amphetamine-induced hyperthermia is believed to be due to dopamine receptor activation and not excessive motor movement (Callaway and Clark, 1994). Amphetamine treatment in mice has been shown to increase liver and brain levels of a 70 kDa heat shock protein (hsp70i), presumably as a result of increased body temperature (Nowak, 1988). The induction of hsp70i by amphetamine is similar to that observed in cultured cells in which the media temperature is raised 3-5°C above normal culture temperature, which causes induction of a variety of heat shock proteins (hsps; for reviews see Parsell and Lindquist, 1994; Voellmy, 1994; Welch, 1992).

Many studies have documented that increased levels of hsps in cultured cells induced by mild hyperthermic treatment can result in tolerance to severe hyperthermia and other stressors including chemical inducers of hsps such as arsenic and diamide (Kampinga et al, 1995; Li and Werb, 1982; Hahn and Li, 1982). It has been proposed that the apparent ability of hsps to provide protection from various adverse stimuli in these experiments is due to their ability to bind non-native proteins (Palleros et al., 1991; Hightower et al., 1994) and chaperone their refolding or elimination. Only a handful of

studies have addressed the question whether hsps provide protection from various insults in whole animals (Villar et al., 1993; Hotchkiss et al., 1993; Currie et al., 1988; Saad et al., 1995), in part because of the difficulty of inducing hsps in vivo. In such studies, hyperthermia was typically produced by heating animals in incubators or water baths. The animals often had to be anesthetized, however, and a large variability in the response of the animals was noted (Myers et al., 1992; DeMaio et al., 1993; Blake et al., 1990; Abe et al., 1993). Amphetamine treatment offers a novel alternative to increase hsps in a variety of tissues, including the liver.

The first goal of this study was to confirm and extend the previous observation that hyperthermic treatment by amphetamine administration increases hsp levels in mice, specifically in liver tissue. The second objective was to determine whether increases in hepatic hsps resulting from amphetamine treatment are associated with a diminished susceptibility to hepatotoxicants. To test this, mice with elevated hsps from amphetamine treatment were challenged with one of four different hepatotoxicants – acetaminophen, bromobenzene, carbon tetrachloride, or cocaine. The effects of amphetamine pretreatment on liver injury, hepatotoxicant bioactivation and hepatic glutathione status were assessed.

## Materials and Methods

Animals and treatments. Adult ICR male mice (Harlan Sprague-Dawley, Indianapolis, IN) weighing 25-30 g were housed on corn cob bedding in temperature- and humidity-controlled animal quarters with a 12-h light/dark cycle. The animals were allowed free access to food and water before and during the experiments, with the exception of the fasting of mice for 16 hours before acetaminophen (APAP) treatment. ACS grade bromobenzene or carbon tetrachloride (Fisher Scientific, Orlando, FL) was administered i.p. in corn oil with an injection volume of 5 ml/kg body weight. APAP (4'-hydroxyacetanilide), D-amphetamine sulfate, and cocaine hydrochloride (Sigma Chemical

Co., St. Louis, MO) were administered i.p. in saline with an injection volume of 10 ml/kg body weight.

For most experiments, mice were pretreated with amphetamine (15 mg/kg) or saline and returned to the animal quarters, which were maintained at 24-25°C. Seventy-two hours after pretreatment, mice were administered one of the four hepatotoxicants (APAP, bromobenzene, carbon tetrachloride, or cocaine) and liver injury was assessed through measurement of serum ALT activity and by histopathology 24, 48, and 72 hours after the dose. In an additional experiment, mice were administered APAP or bromobenzene 144 hours after the amphetamine treatment and liver injury assessed as mentioned above. Animals were killed by carbon dioxide asphyxiation.

Rectal temperatures were measured with a digital thermometer using a YSI 400 Series rectal thermistor probe (Fisher Scientific, Orlando, FL).

Polyacrylamide gel electrophoresis, protein blotting and immunostaining. Sodium dodecyl sulfate- polyacrylamide gel electrophoresis and Western blotting of liver protein was as described previously (Salminen et al., 1997). Briefly, 200 μg of total liver protein from each sample was resolved by electrophoresis on a 12.5% SDS-PAGE gel. The proteins separated by SDS-PAGE were immediately blotted to Hybond-ECL Western membrane (Amersham, England) and then blocked in TBS (20 mM Tris, 500 mM sodium chloride, pH 7.5) containing 3% gelatin. After blocking, the membrane was washed in TTBS (TBS containing 0.05% polyoxyethylenesorbitan monolaurate) and then probed with antibodies to the various hsps (StressGen, Victoria, B.C., Canada) diluted 1:1000 in TTBS containing 1% gelatin. Incubation was for 18 hours at 24°C with continuous shaking. The membrane was washed with TTBS and incubated with continuous shaking for one hour at 24°C with a horseradish peroxidase-conjugated secondary antibody (Amersham, England) at a 1:3000 dilution in TTBS containing 1% gelatin. Next, the membrane was washed in TTBS, the chemiluminescent horseradish peroxidase substrate Luminol (Amersham,

England) was added, and the membrane was exposed to standard X-ray film to localize antibody binding.

Immunohistochemical detection of hsp25 and hsp70i. Immunohistochemical detection of hsp25 and hsp70i accumulation in the liver was as described previously (Salminen et al., 1997). Briefly, sections 4-6 μm thick were cut from formalin-fixed, paraffin-embedded liver specimens. Paraffin embedded sections were deparaffinized and endogenous peroxidase activity was quenched by submerging the slides in 3% hydrogen peroxide containing 0.1% sodium azide. Slides were incubated with blocking solution (TBS containing 25% v/v bovine serum plus 3% w/v purified bovine serum albumin (BSA)) for one hour at 37°C and then washed with TBS. In the case of slides subsequently probed for hsp70i, Fab fragment goat anti-mouse IgG (H+L) (Jackson Immuno Research Laboratories, Inc., West Grove, Pennsylvania) was added to the blocking solution (10 μg/ml final concentration). After the blocking step, slides were incubated with anti-hsp25 antibody (rabbit polyclonal, Stressgen, B.C., Canada) diluted 1:100 in blocking solution or anti-hsp70i (mouse monoclonal, Stressgen, B.C., Canada) diluted 1:100 in blocking solution [devoid of the Fab fragment goat anti-mouse IgG antibody] for one hour at 37°C and then for 18 hours at 24°C. The slides were washed in TBS and then incubated for 30 minutes at 37°C with a biotinylated secondary antibody diluted 1:500 in TBS containing 3% BSA. The sections were washed again and then incubated for 30 minutes at 37°C with streptavidin-linked horseradish peroxidase diluted 1:200 in TBS containing 3% BSA. Finally, the sections were washed and incubated for 15 minutes with the horseradish peroxidase colorimetric substrate 3,3'-diaminobenzidine (DAB) (Sigma Chemical Co., St. Louis, MO) supplemented with 0.03% $NiCl_2$ (w/v). The sections were then counterstained with hematoxylin and dehydrated with graded alcohols and then xylene.

Radiolabeled toxicant binding to protein. Ring-labeled [$^{3}$H]-APAP (Dupont NEN, Boston, MA), [$^{14}$C]-carbon tetrachloride (Dupont NEN, Boston, MA), ring-labeled

[$^{14}$C]-bromobenzene (ICN Radiochemicals, Irvine, CA) or tropine ring-labeled [$^{3}$H]-cocaine hydrochloride (National Institute on Drug Abuse, Rockville, MD) were used to measure covalent binding of toxicant to proteins. Radiolabeled toxicant was added to unlabeled toxicant such that each animal received 3 μCi at the desired total toxicant dose (50 mg/kg cocaine, 0.04 ml/kg carbon tetrachloride, 350 mg/kg APAP, and 0.45 ml/kg bromobenzene, respectively). At varying times after the dose, the mice were killed and the livers removed, rinsed in saline, weighed, and transferred to 15 ml conical tubes on ice containing 5 ml of 6% (w/v) trichloroacetic acid and 1 mM [ethylenedinitrilo]tetraacetic acid. Liver was homogenized on ice and a 0.5 ml aliquot of the homogenate was transferred to a 1.5 ml centrifuge tube for measurement of covalent binding of radiolabeled toxicant to protein. The remaining 4.5 ml of homogenate were used to assess glutathione (GSH) levels as described below. The solution in the 1.5 ml centrifuge tube was sonicated with an Ultratip Labsonic® sonicator (Lab-Line Instruments, Inc., Melrose Park, IL) for 10 seconds at 30-40 watts and the protein pelleted by centrifugation at 8000 x g for 5 minutes. The pellet was washed extensively with 1 ml aliquots of methanol/ether (3:1) until [$^{14}$C]- or [$^{3}$H]-radioactivity in the supernatant was indistinguishable from background. The pellet was air dried and resuspended in 1 N sodium hydroxide. Radioactivity in each sample was determined by liquid scintillation spectrometry. The protein concentration of each sample was measured as described below and used to normalize detected radioactivity to protein content.

GSH depletion. Hepatic GSH was measured as total acid soluble thiols according to the method of Ellman (1959). A 4.5 ml aliquot of liver homogenate was centrifuged at 2000 x g at 4°C for fifteen minutes. Eighty microliters of the supernatant solution were added to 2 ml of phosphate buffer (0.1M, pH 8.0), followed by addition of 40 μl of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB: 4 mg/ml in 95% ethanol). The resulting solution was then vortex mixed and allowed to stand for five minutes at room temperature. GSH

was calculated from the absorbance of the solution at 412 nm using an extinction coefficient of 13,100 (Sedlak and Lindsay, 1968).

Serum alanine aminotransferase activity. Blood for the determination of alanine aminotransferase (ALT) activity was collected by cardiac puncture immediately following carbon dioxide asphyxiation 24, 48, or 72 hours after the hepatotoxicant dose. Serum ALT activities were determined by the method of Bergmeyer, et al. (1978) using a commercially available kit (ALT 20; Sigma Diagnostics, St. Louis, MO).

Protein determination. Protein was measured with the Micro Protein Determination assay (Sigma Chemical Co., St. Louis, MO), based on the method of Bradford (1976), using BSA as standard.

Statistical analysis. Serum ALT activity values and hepatic GSH concentrations were analyzed using a one-way ANOVA followed by a Student Neuman-Keuls post-hoc test. Differences among groups were considered significant at $p \leq 0.05$. ALT data were log transformed prior to statistical analysis.

## Results

Initial experiments were conducted to characterize the time course of hyperthermia resulting from a test dose of amphetamine (15 mg/kg, i.p.). Core body temperature in amphetamine-treated mice rose quickly and peaked near 40°C 30 minutes after the amphetamine dose (Figure 5-1). After two hours, the mean core body temperature dropped to a value that was slightly, but not significantly, lower than the pretreatment ("0 time") core body temperature.

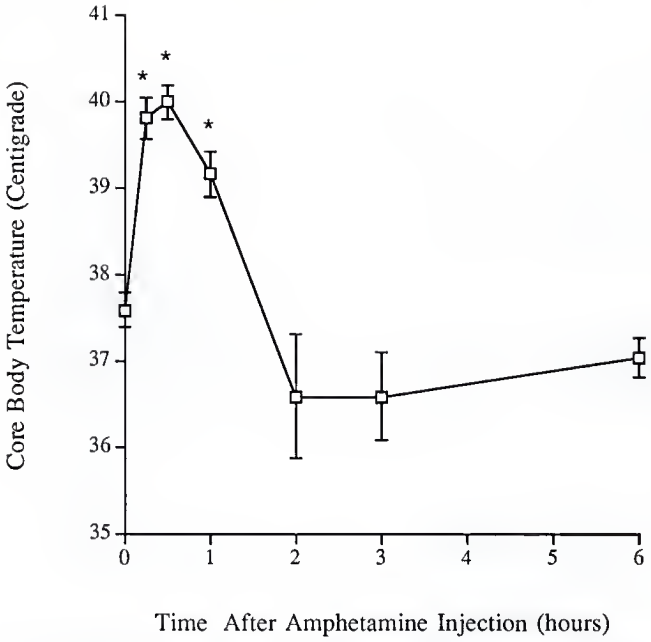



Figure 5-1. Amphetamine-induced hyperthermia. Rectal temperatures were measured at the indicated times after a single dose of amphetamine (15 mg/kg, i.p.). Data are expressed as mean ± SEM (n = 5 mice). * denotes a statistically significant elevation of body temperature above normal body temperature ($p \leq 0.05$).

Amphetamine had been shown to induce hsp70i in murine liver; however, hsp accumulation was only measured two hours after amphetamine treatment (Nowak, 1988). In order to better characterize the time course of hsp accumulation and disappearance in response to amphetamine-induced hyperthermia, mice were euthanized at various intervals from 6 to 144 hours after an amphetamine dose (15 mg/kg, i.p.) and hepatic hsp levels were estimated by SDS-PAGE and Western blot analysis. Amphetamine treatment increased hsp25 and hsp70i levels at 6, 24, 48, and 72 hours, with maximal accumulation of both hsps at 24-48 hours (Figure 5-2). Both hsps returned to pretreatment

(undetectable) levels by 96 hours post-administration in some experiments (Figure 5-2), and by 144 hours in all experiments. The levels of other hsps (hsp60, hsc70, and hsp90) were not altered by amphetamine treatment at any time point. When the animals were housed at 19-20°C instead of the usual 24-25°C, the same 15 mg/kg dose of amphetamine failed to cause hyperthermia. This effect of ambient temperature on amphetamine-induced hyperthermia had been noted previously (Nowak, 1988; Yehuda and Wurtman, 1972). No increase in hsp25 or hsp70i levels following amphetatamine treatment was observed by Western blotting or immunohistochemistry when hyperthermia failed to occur (not shown).

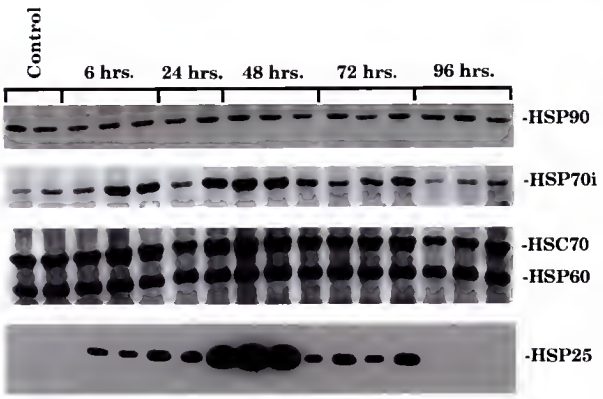
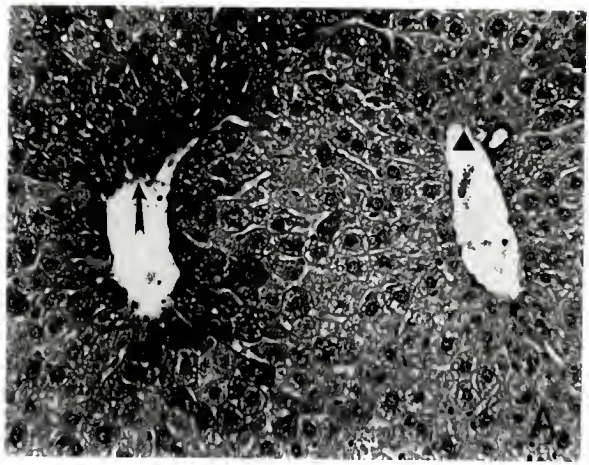
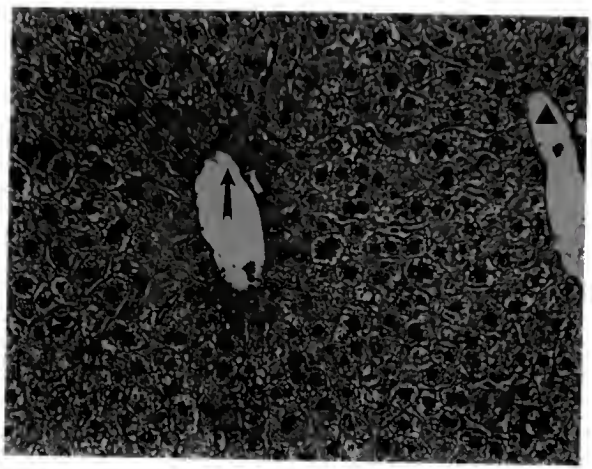



Figure 5-2. Heat shock protein (hsp) induction in murine liver 0, 6, 24, 48, 72, or 96 hours after treatment with amphetamine (15 mg/kg, i.p.). Liver protein was resolved on a 12.5% SDS-PAGE gel, and the levels of various hsps were determined by Western blotting using antibodies specific for the indicated hsps. Equal amounts of protein (i.e., 200 µg) from each sample was loaded onto separate lanes.

Figure 5-3. Immunohistochemical detection of hsp25 and hsp70i accumulation in murine liver after amphetamine (15 mg/kg, i.p.) treatment. Hsp25 and hsp70i accumulation was measured 72 hours after amphetamine treatment. The immunostained slides were counterstained with hematoxylin. Arrow indicates central vein; arrow head indicates portal triad. A) hsp25 accumulation; B) hsp70i accumulation.

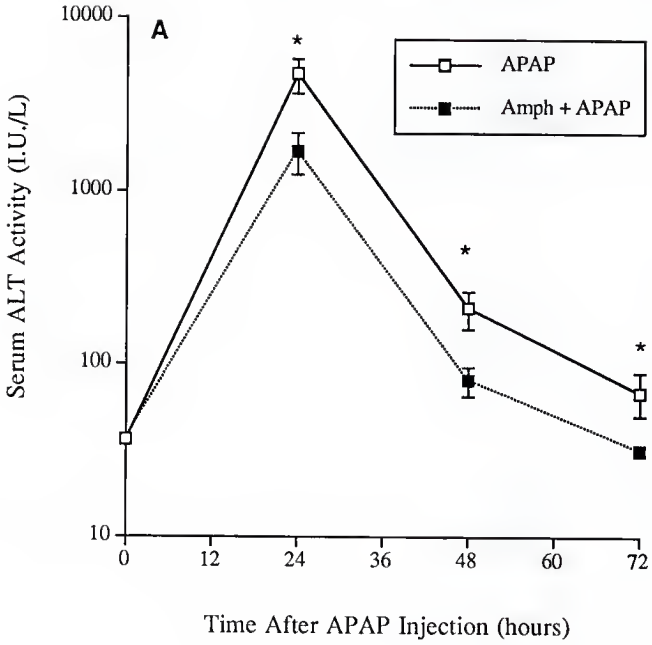


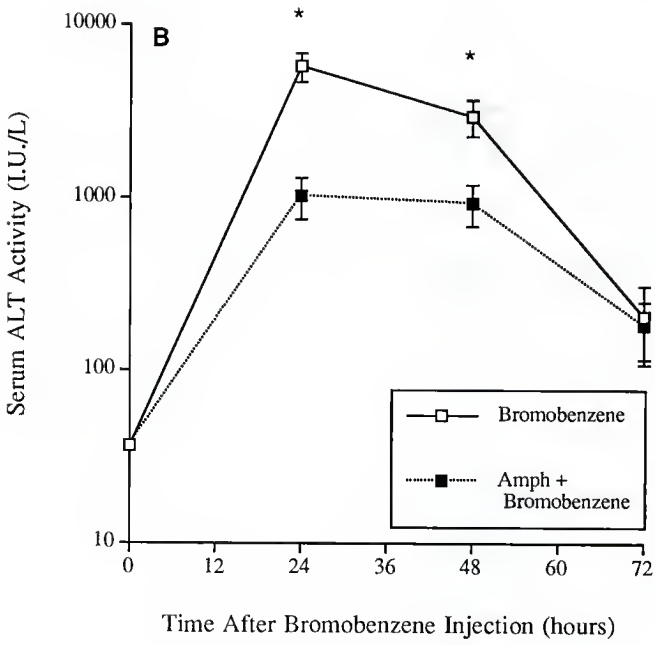


A subsequent series of experiments was conducted to determine whether increased hepatic hsp25 and hsp70i levels from amphetamine treatment are associated with increased resistance or tolerance to hepatotoxicants. To avoid having results confounded by acute effects of amphetamine, hepatotoxicant administration was delayed until 72 hours after the amphetamine dose. This corresponded to the latest time point after amphetamine treatment at which elevated hsp25 and hsp70i levels were observable by the Western blot experiments described above. Immunohistochemical staining for hsps at this time point showed that hsp25 accumulation was restricted to hepatocytes within the centrilobular regions of the liver (i.e., zone 3), while hsp70i was elevated uniformly throughout the lobule (Figure 5-3).

Amphetamine pretreatment markedly decreased hepatic necrosis from bromobenzene and APAP as measured by serum ALT activities (Figure 5-4). Histopathological examination of liver sections taken at 24, 48, and 72 hours corroborated the ALT activity data. Representative sections from bromobenzene- and APAP-treated mice, with and without amphetamine pretreatment, are shown in Figure 5-5. Elevated serum ALT activities from carbon tetrachloride or cocaine were not affected by amphetamine pretreatment (Figure 5-4), and histopathological examination of liver sections confirmed the absence of any protection against hepatotoxicity from these compounds (not shown). As a follow-up experiment, the interval between amphetamine and hepatotoxicant dose was extended to 144 hours, at which time hsp25 and hsp70i levels had returned to normal (see above). Under these conditions, amphetamine pretreatment afforded no protection against the hepatotoxicity of either APAP or bromobenzene (not shown).

Figure 5-4. The effect of amphetamine pretreatment on acetaminophen, bromobenzene, carbon tetrachloride, or cocaine hepatotoxicity. A single dose of saline or amphetamine (15 mg/kg, i.p.) was administered 72 hours prior to hepatotoxicant challenge. Serum alanine aminotransferase activity was assessed at the indicated times after a single dose of A) APAP (350 mg/kg, i.p.), B) bromobenzene (0.45 ml/kg, i.p.), C) carbon tetrachloride (0.04 ml/kg, i.p.), or D) cocaine (50 mg/kg, i.p.) as an indicator of liver injury. Data are expressed as the means ± SEM (n = 8- 15 mice per group). * denotes a statistically significant difference between the two means at a given time point ($p \leq 0.05$). NOTE: y-axis values shown are log normal. ALT= alanine aminotransferase; AMPH= amphetamine; APAP= acetaminophen.


A
10000
1000
100
10
Serum ALT Activity (I.U./L)
APAP
Amph + APAP
*
*
*
0
12
24
36
48
60
72
Time After APAP Injection (hours)


B
10000
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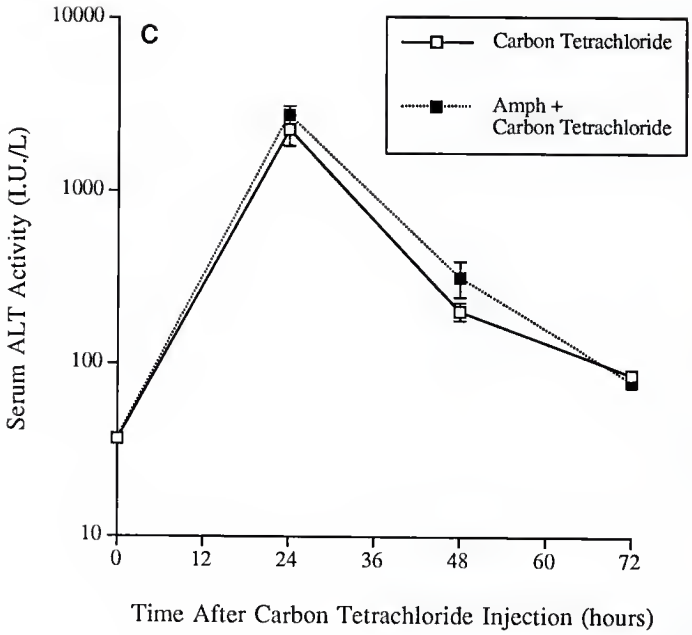
*
Serum ALT Activity (I.U./L)
0
12
24
36
48
60
72
Time After Bromobenzene Injection (hours)
Bromobenzene
Amph + Bromobenzene


C
Carbon Tetrachloride
Amph +
Carbon Tetrachloride
Serum ALT Activity (I.U./L)
10000
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100
10
0
12
24
36
48
60
72
Time After Carbon Tetrachloride Injection (hours)

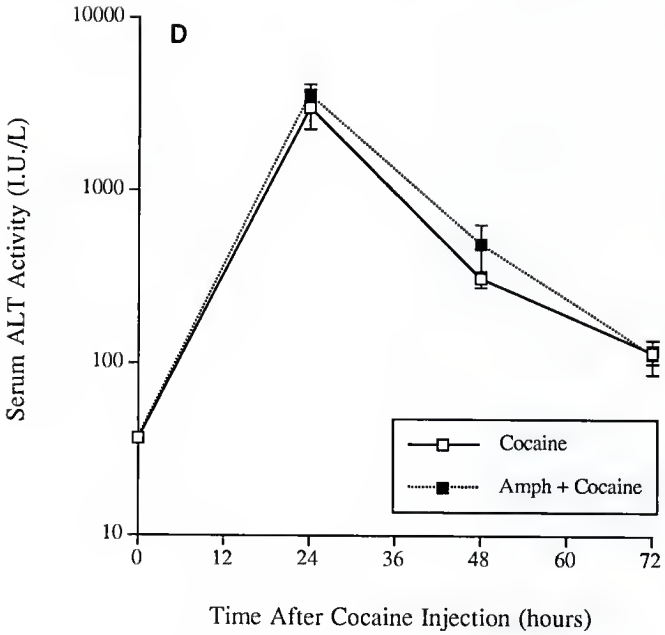
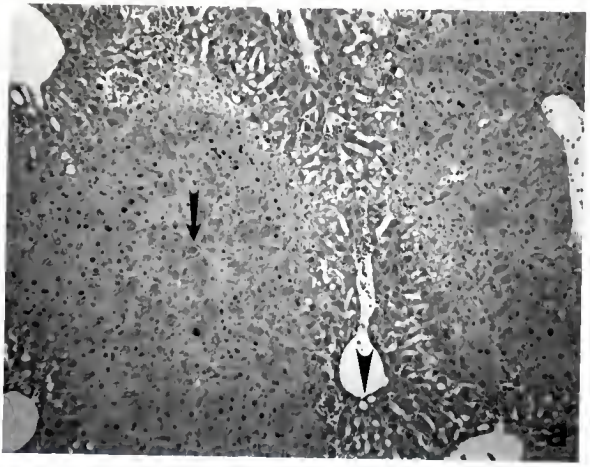

D
10000
1000
100
10
Serum ALT Activity (I.U./L)
0
12
24
36
48
60
72
Time After Cocaine Injection (hours)
Cocaine
Amph + Cocaine

Figure 5-5. Liver histopathology of saline or amphetamine pretreated mice administered a single dose of bromobenzene or acetaminophen. A single acute dose of saline or amphetamine (15 mg/kg, i.p.) was administered 72 hours prior to the bromobenzene (0.45 ml/kg, i.p.) or acetaminophen (350 mg/kg, i.p.) dose. A) saline + bromobenzene, B) amphetamine + bromobenzene, C) saline + acetaminophen, and D) amphetamine + acetaminophen represent typical sections from mice 24 hours after the bromobenzene or acetaminophen dose. Arrow indicates central vein; arrow head indicates portal triad. Sections are stained with hematoxylin-eosin.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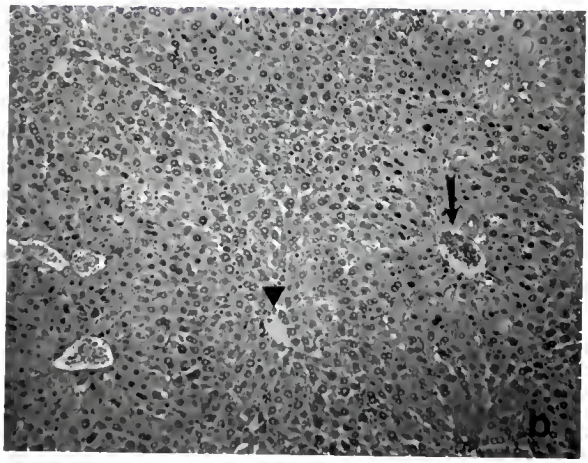
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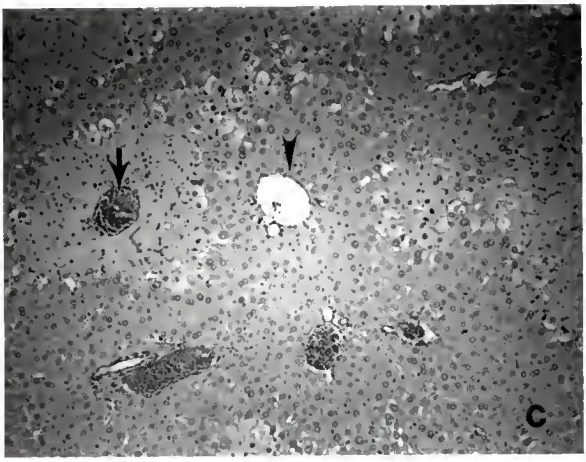
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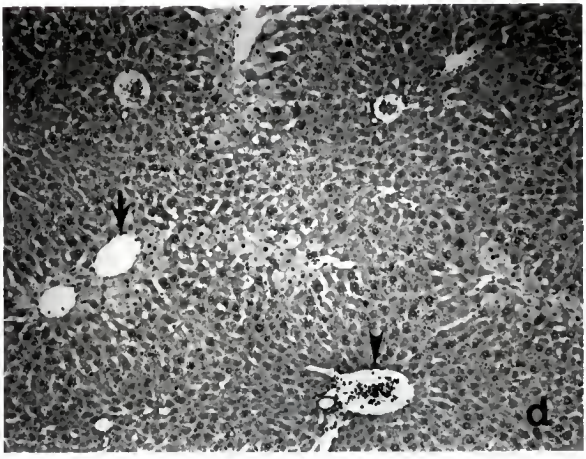
d

In an attempt to explore alternative explanations for the protective effects of a 72-hour amphetamine pretreatment, the extent of reactive metabolite formation and binding was evaluated in experiments in which mice were administered radiolabeled doses of the toxicants and the extent of covalent binding to protein was measured. Previous studies have shown relatively rapid covalent binding to proteins after APAP, carbon tetrachloride, and cocaine administration to mice (Roberts et al., 1995; Evans, 1983; Rashed et al., 1990). Consequently, covalent binding was assessed 1 and 4 hours after administration of these toxicants. Reactive metabolite binding of bromobenzene was known to occur over a more protracted period of time (Casini et al., 1985). Therefore, bromobenzene binding to protein was assessed 6 and 24 hours after the radiolabeled dose. As shown in Table 5-1, the 72-hour amphetamine pretreatment had no appreciable effect on covalent binding of radiolabel from any of the hepatoxicants. The only statistically significant difference was an increase in covalent binding of bromobenzene in amphetamine pretreated mice 6 hours after the dose.

As had been observed previously, covalent binding of APAP and bromobenzene was accompanied by a marked depression in hepatic GSH content, while comparatively little change in GSH concentration resulted from treatment with carbon tetrachloride or cocaine (Table 5-2). Consistent with the absence of an effect on covalent binding, amphetamine pretreatment also had no discernable effect on hepatic GSH status of the liver, before or during hepatotoxicant exposure.

Table 5-1. Irreversible Binding of Hepatotoxicants to Protein.

| Treatment | Duration (hours) | Pretreatment | |
|---|---|---|---|
| | | Saline | Amphetamine |
| [$^{3}$H]-APAP | 1 | 0.64 ± 0.06 | 0.79 ± 0.02 |
| | 4 | 0.82 ± 0.04 | 0.80 ± 0.08 |
| [$^{14}$C]-Bromobenzene | 6 | 1.48 ± 0.06 | 1.98 ± 0.12* |
| | 24 | 1.80 ± 0.26 | 2.20 ± 0.51 |
| [$^{14}$C]-Carbon Tetrachloride | 1 | 0.51 ± 0.09 | 0.37 ± 0.04 |
| | 4 | 0.31 ± 0.01 | 0.29 ± 0.01 |
| [$^{3}$H]-Cocaine | 1 | 0.071 ± 0.012 | 0.049 ± 0.009 |
| | 4 | 0.052 ± 0.009 | 0.071 ± 0.017 |

Note. Mice were pretreated with amphetamine (15 mg/kg, i.p.) or saline vehicle. Seventy two hours after the pretreatment, mice were treated with [$^{3}$H]-acetaminophen (350 mg/kg, i.p.), [$^{14}$C]-bromobenzene (0.45 ml/kg, i.p.), [$^{14}$C]-carbon tetrachloride (0.04 ml/kg, i.p.), or [$^{3}$H]-cocaine (50 mg/kg, i.p.) (3 μCi/animal). Livers were harvested at the indicated times and analyzed for toxicant-protein adduct levels as described in Materials and Methods. Values represent mean ± SEM (n = 3-5 animals). * indicates a statistically significant difference from the value obtained in saline pretreated mice administered the same hepatotoxicant at the same time of analysis ($p \leq 0.05$).

Table 5-2. Liver Glutathione Levels After Hepatotoxicant Exposure.

| Treatment | Duration (hours) | Pretreatment: Saline | Pretreatment: Amphetamine |
|---|---|---|---|
| APAP | 1 | 8.1 ± 0.4 | 7.5 ± 0.3* |
| | 4 | 40.5 ± 5.0 | 46.6 ± 8.4 |
| Bromobenzene | 6 | 9.0 ± 1.2 | 6.2 ± 0.3* |
| | 24 | 108.1 ± 9.8 | 114.8 ± 14.5 |
| Carbon Tetrachloride | 1 | 103.4 ± 8.6 | 94.6 ± 5.0 |
| | 4 | 98.0 ± 9.0 | 88.0 ± 4.0 |
| Cocaine | 1 | 98.2 ± 4.7 | 89.5 ± 4.2 |
| | 4 | 86.3 ± 10.7 | 67.2 ± 1.5 |

Note. Mice were pretreated with amphetamine (15 mg/kg, i.p.) or saline vehicle. Seventy two hours after the pretreatment, mice were treated with one of the indicated hepatotoxicants as described in Table 1. Livers were harvested at the indicated times and analyzed for glutathione levels as described in Materials and Methods. Values represent mean ± SEM (n = 3-5 animals). * indicates a statistically significant difference from the value obtained in saline pretreated mice administered the same hepatotoxicant at the same time of analysis ($p \leq 0.05$).

## Discussion

The results of this study confirm and extend previous observations by Nowak (1988) of increased hsps in the liver of mice made hyperthermic by administration of amphetamine. In the present study, transient amphetamine-induced hyperthermia lasting less than two hours was associated with marked elevations of hsp70i and hsp25 in the liver that persisted for days. While these hsps presumably accumulate in response to hyperthermia, the same two hsps have been shown previously to be increased in mice treated with a number of hepatotoxicants (Voellmy et al., 1994). In this study, no hepatic injury was observed to result from the administration of amphetamine, and it therefore appears unlikely that hsp induction was due to toxicity. The observation that increases in hsp levels did not occur when mice given the same amphetamine dose were housed at a somewhat lower ambient temperature, preventing the development of hyperthermia, lends

additional support to the conclusion that hyperthermia was responsible for the elevated hsps.

Apparently, different hsps are induced by amphetamine in mouse versus rat liver. Lu and Das (1993) found induction of hsp27 and hsp89 mRNA, but not hsp70i mRNA, in rat liver three hours after amphetamine treatment. In contrast, Nowak (1988) found the induction of a 70 kDa protein in mouse liver with characteristics of hsp70i two hours after amphetamine treatment. Our study also found induction of hsp70i in mouse liver, plus induction of hsp25. Nowak (1988) did not detect increased synthesis of proteins with a molecular mass at or near 25-kDa following amphetamine treatment, which is presumably explained by his use of $^{35}$S-methionine for protein labeling and the fact that rodent hsp25 lacks methionine residues (Kim, 1983).

In a previous study using the HepG2 cell line, sub-lethal heat treatment increased hsp70i levels and provided substantial protection against cytotoxicity from subsequent exposure to several hepatotoxicants (Salminen et al., 1996). Cytoprotection appeared to be correlated with the ability of the hepatotoxicant to induce hsp70i in cells without pretreatment. That is, the greatest reduction in cytotoxicity was observed with toxicants that alone produced the greatest increases in hsp70i levels, viz., diethylnitrosamine and cadmium. At the other extreme, cocaine and carbon tetrachloride treatment did not produce measurable increases in hsp70i concentrations, and elevated hsps from mild heat pretreatment offered no cytoprotection from these compounds. Among the chemicals tested in HepG2 cells, three were also evaluated in the present study in amphetamine-pretreated mice. As in HepG2 cells with mild heat pretreatment, hyperthermia from amphetamine pretreatment resulted in reduced toxicity from bromobenzene, but not cocaine or carbon tetrachloride. We have reported previously that treatment with both cocaine and carbon tetrachloride increases hsp70i (and hsp25) levels in mouse liver (Salminen et al. 1997; Roberts et al., 1996), and in this respect the results in vivo are different from those observed in vitro in HepG2 cells. This difference may be due to the comparative absence

in HepG2 cells of the metabolic pathways producing reactive metabolites from these compounds that exist in vivo (Salminen et al., 1996).

While an objective of this study was to examine the mitigating influence of hsps on hepatotoxicity, it is possible that consequences of amphetamine pretreatment other than hsp induction may have contributed to, or been responsible for, the observed cytoprotective effects. The ability of amphetamine and related drugs to influence the hepatotoxicity of other drugs and chemicals has been well documented (Roberts et al., 1997), but toxicity is consistently increased rather than decreased, and has only been observed when the amphetamine (or amphetamine-related drug) and the hepatotoxicant doses were administered within a few hours of each other. In order to minimize the possibility that results are confounded by acute amphetamine effects, treatments with hepatotoxicants were delayed for 72 hours in these experiments. At that time, hsp25 and hsp70i were still elevated, but other, more transient effects were presumably minimal or absent. Perhaps the most obvious mechanism by which amphetamine could have decreased the hepatotoxicity of acetaminophen and bromobenzene would have been by preventing somehow their bioactivation and/or the detoxification of their reactive metabolites. This mechanism does not appear to be operative, as amphetamine pretreatment did not significantly alter the extent of covalent binding of any of the hepatotoxicants, except for an increase in covalent binding of bromobenzene at one time point. Also, there was no evidence that amphetamine could have impaired reactive metabolite conjugation by lowering hepatic GSH content, as has been observed previously with acetaminophen when an amphetamine-related compound (phenylpropanolamine) was administered as a 3 hr pretreatment (James et al., 1993). While an alternative cause cannot be ruled out, hsps appear at this point to be the most plausible mechanism of cytoprotection after amphetamine pretreatment.

Assuming that increased hsp70i and/or hsp25 levels are, in fact, responsible for diminishing hepatotoxicity in these experiments, an important question is why only toxicity from acetaminophen and bromobenzene were reduced, and not that of carbon tetrachloride

and cocaine. Each of these hepatotoxicants is similar in that it is converted by cytochrome P-450 to one or more reactive metabolites, and this reactive metabolite formation has been postulated to play a role in their cytotoxic effects (Rashed et al., 1990; Evans, 1983; Hanzlik et al., 1989; Sipes and Gandolfi, 1982). There are, however, differences in the nature of the reactivity of these metabolites. Reactive metabolites of acetaminophen and bromobenzene bind extensively with sulfhydryls, and profound depletion of hepatic glutathione content characteristically occurs at hepatotoxic doses (Rashed et al., 1990; Casini et al., 1985). Hepatotoxic doses of carbon tetrachloride and cocaine, in contrast, produce metabolites that are less sulfhydryl-reactive and result in very little depletion of hepatic glutathione content (Evans, 1983; Recknagel et al., 1989). One possible explanation for differences in the protective effects toward the two sets of toxicants is that hsp25 and hsp70i may be better able to prevent proteotoxicity from adduction of some target proteins than others, and this may be related to target protein sulfhydryl content. Alternatively, increased levels of hsp25 and hsp70i may simply serve as alternative protein targets for reactive metabolites of acetaminophen and bromobenzene (but not cocaine and carbon tetrachloride), sparing proteins critical for cell function from adduction. Yet another possibility is that the difference in cytoprotection from hsp induction is a function of the relative importance of proteotoxicity in the overall cytotoxic effects of the chemical. Given the role of hsps in binding and refolding denatured proteins, it seems reasonable to suspect that hsps primarily provide protection against proteotoxicity. If other mechanisms, such as lipid peroxidation, are more important in producing cell injury from a chemical, the ability of hsp induction to diminish toxicity will be limited. In this regard, it is worth noting that lipid peroxidation is a prominent feature of liver injury from both cocaine and carbon tetrachloride (Kloss et al., 1983; Yao et al., 1994), but not for acetaminophen. Further experimentation will be required to determine which, if any, of these potential mechanisms is responsible for differences in cytoprotection apparently mediated by hsps in liver in vivo.

# CHAPTER 6
# EFFECT OF N-ACETYL-CYSTEINE ON HEAT SHOCK PROTEIN INDUCTION BY ACETAMINOPHEN IN MOUSE LIVER: THE ROLE OF PROTEIN ARYLATION

## Introduction

Heat shock proteins (hsps) are ubiquitous proteins expressed in both prokaryotic and eukaryotic cells in response to a variety of stressors (for reviews see: Lindquist and Craig, 1988; Welch, 1992; Voellmy, 1994; Parsell and Lindquist, 1994). Recently, we have shown that acetaminophen (APAP), bromobenzene, carbon tetrachloride, and cocaine induce hsp25 and hsp70i in mouse liver (Salminen et al., 1997; Roberts et al., 1996; Chapter 4 of this dissertation). All of the previously mentioned toxicants are metabolized in the liver into reactive intermediates that covalently bind protein (Plaa, 1993; Lindamood, 1991; Evans, 1983). It is conceivable that adducted proteins resulting from reactive metabolite binding are physiologically equivalent to proteins unfolded as a consequence of exposure to classical inducers of the stress response (e.g., heat, heavy metals, etc.), thereby activating hsp gene expression. This hypothesis would be consistent with a number of observations suggesting that stress induction of hsp synthesis is mediated by the presence of non-native proteins. For example, overexpression of mammalian proteins unable to fold properly causes activation of hsp genes in bacteria (Goff and Goldberg, 1985), and the injection of chemically-denatured proteins into vertebrate cells increases stress protein synthesis while injection of the corresponding native protein does not (Ananthan et al., 1986).

The induction of hsps by toxicant adducted protein was eluded to by studies comparing the intralobular pattern of binding of APAP and cocaine to protein with hsp induction using immunohistochemistry. For both toxicants, hsp induction was restricted to

hepatocytes that contained covalently bound toxicant suggesting that toxicant binding and hsp induction were related (Salminen et al., 1997; Chapter 4 in this dissertation). In addition, elimination of cocaine hepatotoxicity with a cytochrome P-450 inhibitor prevented toxicity and hsp induction (Salminen et al., 1997). Cytochrome P-450 inhibitors prevent the covalent binding of many toxicants to protein by inhibiting their metabolism into reactive intermediates, but they also inhibit other aspects of toxicity; therefore, the effect of toxicant adduction of protein in triggering hsp induction can not be separated from other aspects of toxicity using inhibitors of metabolism. Some hepatoprotective compounds can completely prevent toxicity without affecting toxicant adduction of protein (i.e., covalent binding and toxicity can be separated) (Yao et al., 1994; Casini et al., 1985). These compounds provide unique tools to determine the role toxicant adduction of protein plays in triggering hsp induction.

N-acetyl-cysteine (NAC; Mucomyst®) is a hepatoprotective compound that is the primary treatment for APAP overdose in humans (Prescott, 1983). Gerber et al. (1977) showed that NAC prevented APAP hepatotoxicity without affecting the arylation of protein by APAP (i.e., NAC did not inhibit the metabolism of APAP into a reactive intermediate and/or directly scavenge the reactive metabolites). Corcoran et al. (1985) provided evidence suggesting that NAC did inhibit APAP arylation of protein and concluded that previous reports showing no affect of NAC on APAP binding were skewed due to influx of extra-hepatic protein and fluid into the livers of APAP treated, but not APAP + NAC treated mice. Using Western blot detection of acetaminophen adduction of protein we show in this report that NAC does not inhibit the binding of APAP to protein when administered 1 or 3 hours after the APAP dose and inhibits binding only 52% when administered simultaneously with APAP. We used this ability of NAC to determine if APAP adducted protein could trigger hsp induction independently of toxicity.

## Materials and Methods

Animals and treatments. Adult B6C3F1 male mice (Harlan Sprague-Dawley, Indianapolis, IN) weighing 25-28 g were used. Mice were housed on corn cob bedding in temperature- and humidity-controlled animal quarters with a 12-h light/dark cycle, and allowed free access to water before and during the experiments. Mice were fasted for 16 hours before the APAP (4'-hydroxyacetanilide) (Sigma Chemical Co., St. Louis, MO) dose and then given food for the duration of the experiments. Mice were administered a single i.p. dose of 250 mg/kg APAP in warm saline. Some mice were treated at 0 (simultaneuosly), 1, or 3 hours after the APAP dose with NAC (300 mg/kg, i.p.) dissolved in saline. Diallyl sulfide, 200 mg/kg p.o., was dissolved in corn oil and administered 2, 24, and 48 hours before treatment with APAP to inhibited cytochrome P-450 2E1 activity. APAP and NAC were administered with an injection volume of 10 µl/g body weight and diallyl sulfide was administered with an injection volume of 5 µl/g body weight. Mice were killed by carbon dioxide asphyxiation.

Polyacrylamide gel electrophoresis, protein blotting, and immunostaining. SDS-PAGE and Western blot detection of hsp25 and hps70i levels in total liver protein was as described previously (Salminen et al., 1997). The level of APAP arylation of protein was measured using the same Western blotting protocol used to detect hsp levels. Briefly, an antibody to APAP that has been characterized previously (Matthews et al., 1996) was diluted 1:1000 in TTBS (20 mM Tris, 500 mM sodium chloride, 0.05% polyoxyethylenesorbitan monolaurate, pH 7.5) containing 1% gelatin. Antibodies to the 55 and 100kDa acetaminophen binding proteins (rabbit polyclonal antibodies) were used to determine the level of these proteins in each sample as a loading control. These antibodies were used at a dilution of 1:5000 in TTBS containing 1% gelatin. The level of actin in each sample was determined by Western blotting using an affinity purified antibody ([Product

Number] Sigma Diagnostics, St. Louis, MO) diluted 1:1000 in TTBS as another loading control. All antibodies were incubated with the membranes at 24°C for 18 hours. The primary antibody binding was detected using an alkaline phosphatase-conjugated secondary antibody followed by incubation with the alkaline phosphatase colorimetric substrate BCIP/NBT (100 mM Tris-base, pH 9.5, 165 µg/ml 5-bromo-4-chloro-3-indolyl phosphate, 330 µg/ml nitro blue tetrazolium, 100 mM sodium chloride, 5 mM magnesium chloride).

Densitometry. Western blots probed for APAP arylation of protein were digitized at 150 dots per inch using a desktop computer scanner. The total signal produced by the anti-APAP antibody for each sample was measured from 200 kDa down to 20 kDa using the NIH Image (Version 1.52) computer program. The total density of all the protein bands from 200 to 20 kDa was used as an indicator of total binding of APAP to protein.

Glutathione analysis by high performance liquid chromatography (HPLC). Reduced glutathione (GSH) levels in liver after NAC and/or APAP treatment were measured by HPLC using electrochemical detection. At varying times after the NAC and/or APAP dose, the mice were killed and the livers removed, rinsed in saline, weighed, and transferred to a 15 ml conical tube containing 5 ml of 2% perchloric acid on ice. The livers were homogenized on ice and the precipitated-protein pelleted by centrifugation at 2000 x g for 5 minutes at 4°C. Five hundred microliters of the supernatent was removed and filtered by centrifugation through a 2 ml, 0.22 µm spin filter (Whatman Lab Sales, Hillsboro, Oregon). Samples were stored at -80°C until processing. Each sample was diluted 1:100 in mobile phase (0.1 M monochloroacetic acid, 1 mM sodium octyl sulfate, pH 3.0) and loaded onto the HPLC for determination of GSH levels. A Bioanalytical Systems, Inc. HPLC (Bioanalytical Systems, Inc., West Lafayette, IN) with a mercury-gold amalgam dual-electrode was used for the detection of biomolecules containing sulfhydryl groups. The upstream and downstream electrode potentials were -1.0 and 0.15 volts, respectively. A Suplex pKb 100 reversed-phase column (15 cm length, 4.6 mm ID,

5 µm; Supelco, Bellefonte, PA) was used to separate GSH from other sulfhydryl-containing biomolecules. Mobile phase flow rate was 1.7 ml/min and the mobile phase was constantly purged with nitrogen to displace any dissolved oxygen. The sample volume loaded onto the column was 50 µl. A standard curve of GSH was prepared daily and a one point standard (5 µg/ml) was subsequently run before each set of samples comprising a given treatment group harvested at the same time. Using HPLC allowed separation of cysteine and NAC from GSH since the retention times were typically 1.4, 2.0, and 2.5 minutes, respectively. The limit of detection of GSH was 0.5 µg/ml.

Protein determination. Protein was measured with the Micro Protein Determination assay (Sigma Chemical Co., St. Louis, MO) using BSA as standard.

Serum alanine aminotransferase (ALT) activity. Blood for measurement of serum ALT activity was collected by cardiac puncture immediately after carbon dioxide asphyxiation. Serum ALT activity was determined according to the method of Bergmeyer et al. (1978) using a commercially available kit (Sigma Diagnostics, St. Louis, MO).

Statistical analysis. Data were analyzed by a one-way ANOVA followed by a Student Neuman-Keuls post-hoc test. The level of significant difference was defined as the 0.05 level of probability. ALT data were log transformed prior to statistical analysis.

## Results

Our previous report showed that APAP induced only hsp25 and hsp70i without affecting the level of hsp60, hsc70, and hsp90 (Chapter 4 of this dissertation). In addition, a non-hepatotoxic dose of APAP (i.e., a dose that did not cause necrosis within 24 hours) failed to induce any hsp. Therefore, the current study focused on the ability of a hepatotoxic dose of APAP (250 mg/kg, i.p.) to induce hsp25 and hsp70i with and without NAC (300 mg/kg, i.p.) treatment. Preliminary experiments measured the ability of NAC to inhibit APAP hepatotoxicity as measured by serum alanine aminotransferase (ALT) activity and histopathological changes (i.e., cell swelling and/or necrosis) as observed by light

microscopy. NAC administered at the same time as the APAP dose (0 hr.) completely prevented APAP hepatotoxicity as measured by serum ALT activity (Figure 6-1) and histopathology (not shown). APAP hepatotoxicity was dramatically reduced by NAC administered one hour after the APAP dose with no significant effect of NAC on APAP hepatotoxicity when administered three hours after the APAP dose (Figure 6-1).

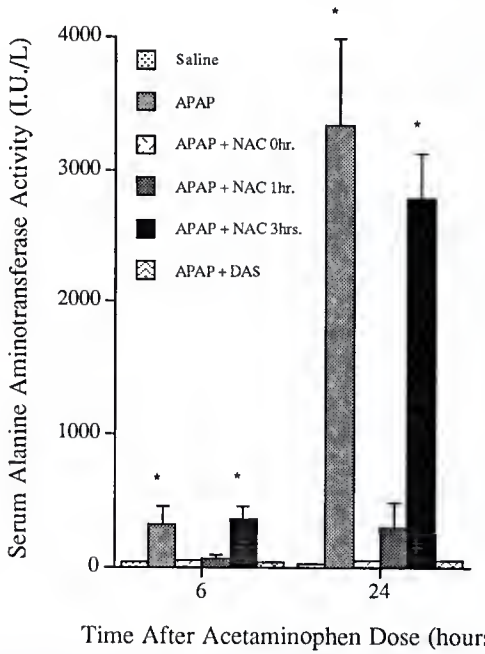


Figure 6-1. The effect of N-acetyl-cysteine (NAC) or diallyl sulfide (DAS) on acetaminophen (APAP) hepatotoxicity. Mice were administered a single dose of APAP (250 mg/kg). NAC (300 mg/kg) was administered 0, 1, or 3 hours after the APAP dose and DAS (200 mg/kg) was administered 2, 24, and 48 hours before the APAP dose. Serum alanine aminotransferase activity (ALT) was assessed 6 or 24 hours after the APAP dose as an indicator of liver injury. Saline, NAC only, and DAS only treated mice had similar serum ALT levels (30 - 40 I.U./L); therefore, their values are not plotted. Data are expressed as the means ± SEM (n = 6 - 9 mice per treatment at each time). * denotes a statistically significant decrease in serum ALT levels compared to APAP only treated mice ($p \leq 0.05$).

Previous studies measured the effect of NAC on APAP arylation using radiolabeled APAP (Gerber et al., 1977; Corcoran et al., 1985). We used Western blot detection of APAP arylation to determine whether or not NAC effected APAP arylation of protein since the level of overall arylation and the arylation of specific proteins can be measured. Administration of NAC 1 or 3 hours after the APAP dose did not inhibit the binding of APAP to cellular protein at 6 or 24 hours (Figures 6-2 and 6-3). NAC administered at the same time as APAP did reduce APAP binding by 52 and 43% at 6 and 24 hours, respectively (Figures 6-2 and 6-3).

Corcoran et al. (1985) suggested that toxicant covalent binding measurements are often underestimated since many toxicants cause liver damage followed by protein and fluid infiltration that skews the final binding estimate when calculated on a protein weight basis. Therefore, several loading controls were used in this study to determine if the amount of protein and fluid infiltrating the liver during APAP exposure significantly affects the amount of liver protein that is loaded onto the gel. If significant infiltration of non-liver protein and fluid occurs then the level of endogenous liver proteins should be less in highly damaged liver (i.e., APAP treated) experiencing edema versus undamaged liver (i.e., APAP + NAC treated). The level of three different proteins were used to determine if significant infiltration of exogenous protein occurs during APAP treatment. The proteins were actin and two cytosolic acetaminophen binding proteins (ABP, 55 and 100 kDa) that APAP preferentially adducts. The level of the three proteins were unaffected by APAP or APAP + NAC treatment at 6 or 24 hours (Figure 6-4). In addition, coomassie blue staining of a duplicate SDS-PAGE gel indicated no differences in the amount of individual proteins loaded (not shown). Therefore, NAC treatment provided a unique tool with which to determine the role APAP binding to protein plays in triggering hsp induction since significant amounts of APAP arylation were present even when NAC was administered at the same time as APAP.

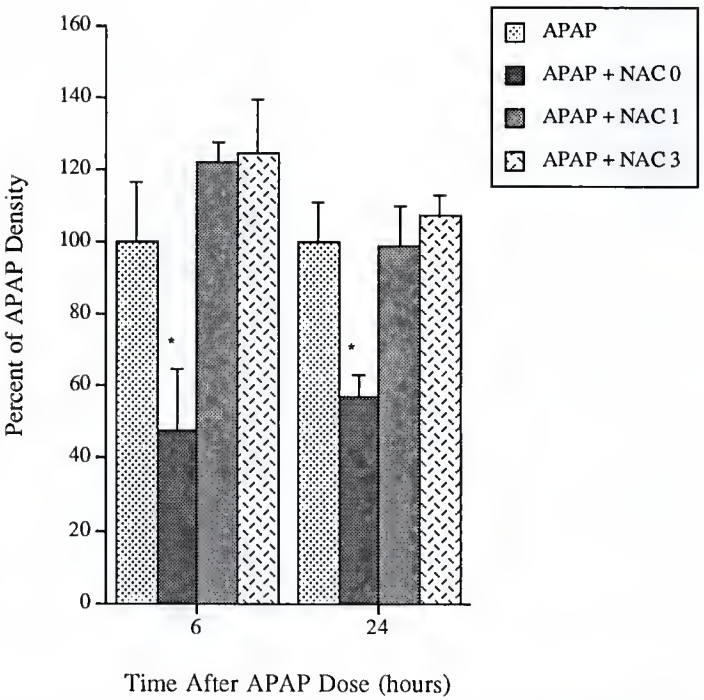


Figure 6-2. Effect of N-acetyl-cysteine (NAC) on acetaminophen (APAP) arylation of protein 6 and 24 hours after treatment with 250 mg/kg APAP. NAC (300 mg/kg) was administered 0, 1, or 3 hours after the APAP dose. Liver samples were collected 6 or 24 hours after the APAP dose. Two hundred micrograms of total liver protein from each sample was resolved on a 12.5% SDS-PAGE gel and the level of APAP arylation of protein was measured by Western blotting using an anti-APAP antibody. The total density of a given lane (i.e., a sample from a single mouse) was measured from 200 kDa down to 20 kDa as described in Material and Methods as an indicator of total APAP arylation of protein. Data are expressed as the means ± SEM (n = 3 mice per treatment at each time). * denotes a statistically significant decrease in binding compared to APAP only treated mice ($p \leq 0.05$).

The effect of NAC treatment on APAP-induced accumulation of hsp25 and hsp70i was measured 6 and 24 hours after the APAP dose by Western blot analysis. These time points were chosen since our previous study noted induction of both hsps at 6 hours with maximal induction at 24 hours (Salminen et al., 1996b). NAC administered 0, 1, or 3 hours after the APAP dose did not prevent the induction of either hsp (Figure 6-4). To determine if blocking APAP arylation of protein would also block hsp induction, pretreatment with diallyl sulfide (DAS, a cytochrome P-450 2E1 inhibitor) was used to block cytochrome P-450 mediated APAP metabolism. DAS inhibited APAP toxicity as measured by serum ALT activity (Figure 6-1) and histopathology as observed by light microscopy (not shown). DAS pretreatment also dramatically reduced APAP arylation of protein and hsp induction at 6 and 24 hours after the dose (Figure 6-5).

Since NAC might prevent toxicity simply by increasing the level of GSH, the effect of NAC on GSH levels was measured. Administration of NAC to naive animals did not alter the level of GSH over time (Figure 6-6). NAC given simultaneously as APAP did not prevent APAP-induced depletion of GSH; however, it did increase the rate of recovery to control levels (Figure 6-6). NAC given one hour after the APAP dose produced a prompt increase in GSH levels to control levels by one hour after the NAC dose increasing to 221% of control levels by 6 hours.

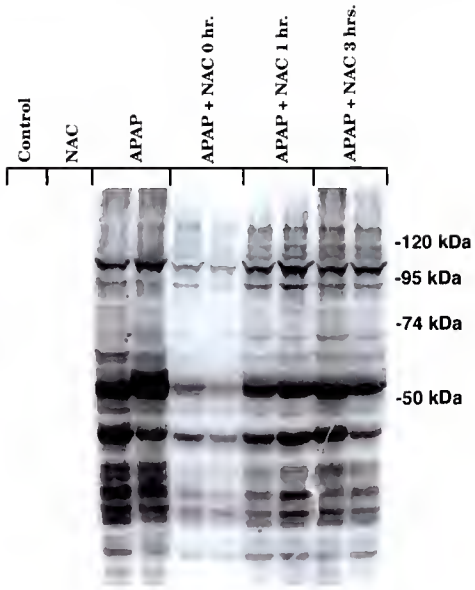


Figure 6-3. Effect of N-acetyl-cysteine (NAC) on acetaminophen (APAP) arylation of protein 24 hours after treatment with 250 mg/kg APAP. NAC (300 mg/kg) was administered 0, 1, or 3 hours after the APAP dose. Total liver protein was resolved on a 12.5% SDS-PAGE gel, and the level of APAP arylation was measured by Western blotting using an anti-APAP antibody. The Western blot is from a single experiment with each lane containing a liver sample from a single mouse. Equal amounts of protein (i.e., 200 μg) from each sample was loaded onto separate lanes.

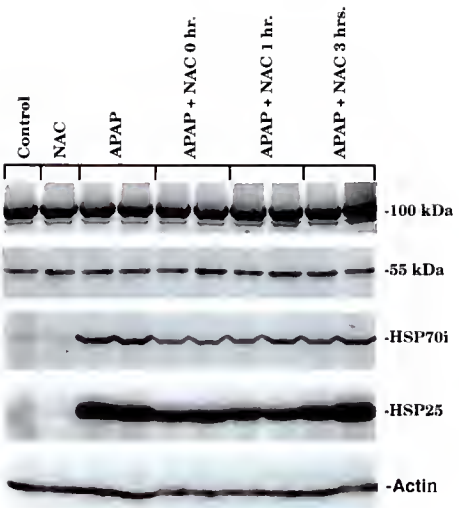


Figure 6-4. Effect of N-acetyl-cysteine (NAC) on acetaminophen (APAP) induction of hsp25 and hsp70i in mouse liver 24 hours after treatment with 250 mg/kg APAP. NAC (300 mg/kg) was administered 0, 1, or 3 hours after the APAP dose. Total liver protein was resolved on a 12.5% SDS-PAGE gel, and the level of hsp25 and hsp70i determined by Western blotting using an antibody specific for the indicated hsp. The level of actin and two APAP binding proteins of 55 and 100 kDa were also measured using antibodies specific for the indicated protein. The levels of these proteins were used as loading controls to ensure that influx of extrahepatic protein did not significantly alter the level of endogenous liver proteins. The liver samples in each lane are the same samples used for Figure 6-3 (e.g., lane 1 of each figure is from the same mouse). Equal amounts of protein (i.e., 200 μg) from each sample was loaded onto separate lanes.

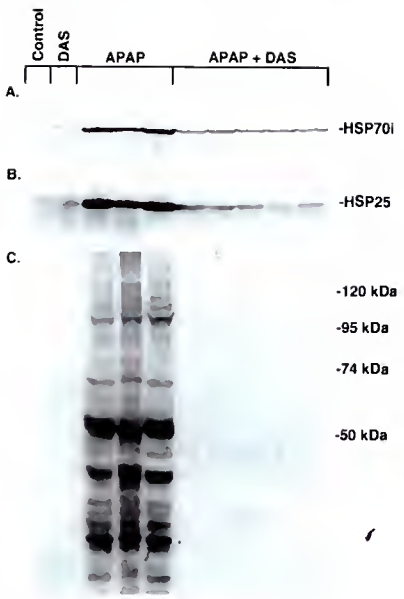


Figure 6-5. Effect of diallyl sulfide (DAS) on acetaminophen (APAP) arylation of protein and induction of hsp25 and hsp70i in mouse liver 24 hours after treatment with 250 mg/kg APAP. DAS (200 mg/kg) was administered 2, 24, and 48 hours before the APAP dose. Protein binding and hsp25 and hsp70i levels were determined as mentioned in Figures 6-3 and 6-4. The Western blot is from a single experiment with each lane containing a liver sample from a single mouse. Equal amounts of protein (i.e., 200 µg) from each sample was loaded onto separate lanes.

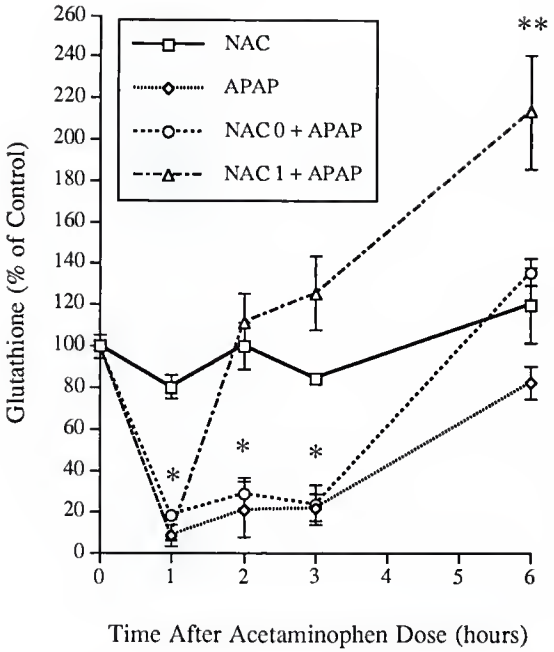


Figure 6-6. The effect of N-acetyl-cysteine (NAC= 300 mg/kg) on the liver levels of glutathione (GSH) with and without acetaminophen (APAP= 250 mg/kg) treatment. NAC was administered 0 or 1 hour after the APAP dose and simultaneously with saline in the NAC only treated mice. Liver levels of GSH were measured 0, 1, 3, or 6 hours after the APAP or saline dose by HPLC using electrochemical detection as described in Materials and Methods. Results are expressed as percentage of the mean NPSH concentration in concurrently euthanized, saline-treated controls. Typical levels of liver GSH in saline-treated controls were 4-5 nmol GSH/mg wet tissue. Data are displayed as the mean ± SEM (n = 3 - 4 mice per treatment at each time point). * denotes a statistically significant decrease compared to control levels for APAP and APAP + NAC 0 treatments. ** denotes a statistically significant increase compared to control levels for APAP + NAC 0 and APAP + NAC 1 treatments ($p \leq 0.05$). Note: APAP + NAC 1 produced a stastically significant decrease in GSH levels one hour after the APAP dose and is the same value as for the APAP only treatment group.

## Discussion

This is the first report of a hepatotoxicant inducing hsps in the whole animal without concurrent or ensuing toxicity. The presented data indicate that covalent binding of APAP to protein is not sufficient to cause hepatotoxicity, but it is able to trigger hsp induction. This correlation must be interpreted with caution, however, since other aspects of toxicity that are not inhibited by NAC may also contribute to hsp induction. Chen et al. (1992) noted that nephrotoxic cysteine conjugates were potent inducers of hsp70 mRNA even if the toxicity, but not the covalent binding, was inhibited by lipid peroxidation inhibitors. They further noted that pretreatment with the sulfhydryl reducing agent dithiothreitol inhibited induction of hsp70 mRNA without affecting the level of covalent binding. They concluded that a combination of protein binding and sulfhydryl oxidation was responsible for hsp induction. In light of our results, it appears that sulfhydryl oxidation may also play a role in triggering hsp induction in the whole animal. NAC did not prevent APAP-induced GSH depletion when administered at the same time as APAP indicating that other sulfhydryl groups in the cell were also probably oxidized. The role of sulfhydryl oxidation can not be conclusively addressed, however, since NAC can also act as a sulfhydryl reducer through disulfide exchange even though it is not as potent of a reducer as a compound such as dithiothreitol (Matthews et al., 1993).

Even though overall protein denaturation is believed to trigger hsp induction, the actual lesion (i.e., protein(s)) that causes induction is unknown. This study provides further support that toxicant adduction of protein can trigger hsp induction. Since NAC did not alter the pattern of APAP arylation of protein (i.e., it did not shift binding to previously unaffected proteins) it can be deduced that one of the proteins that APAP arylates is probably responsible for hsp induction. This was eluded to by our previous study in which a strong correlation existed between hepatocytes containing APAP reactive metabolite and those that had hsp25 and hsp70i accumulation, as measured by

immunohistochemical analysis. In that report we also showed that adduction of protein does not necessarily trigger hsp induction since AMAP (a regioisomer of APAP) was unable to trigger hsp induction despite the fact that it covalently bound protein (Chapter 4 of this dissertation). AMAP has been shown to bind to a lesser extent to organelles more proximal from the site of metabolism; therefore, it appears that one of the proteins that APAP adducts and that AMAP fails to bind may be responsible for hsp induction. It is also possible that oxidation of protein sulfhydryl groups by APAP triggers hsp induction since APAP has been shown to cause protein-sulfhydryl oxidation of mouse liver protein (Tirmenstein and Nelson, 1990). This would be consistent with the GSH data presented since significant GSH depletion occurred even when NAC was administered at the same time as APAP indicating that other sulfhydryl groups were probably oxidized in hepatocytes.

A minor induction of hsp25 and hsp70i was evident in APAP treated mice pretreated with DAS, but the induction was dramatically less than for APAP alone (see Figure 6-5). APAP has been shown to be metabolized to the reactive intermediate NAPQI by the cytochrome P-450 isozyme 2E1 (Lee et al., 1996). Since DAS is a potent 2E1 inhibitor, DAS pretreatment would be expected to decrease the formation of NAPQI from APAP and diminish APAP-induced hepatotoxicity and binding to protein which were observed (see Figures 6-1 and 6-4, respectively). These data further indicate that covalent binding of APAP to protein is important in triggering hsp induction since decreasing APAP binding almost completely prevented hsp induction. This must be carefully interpreted since DAS also prevents other aspects of APAP toxicity which may trigger hsp induction independently of covalent binding. It is possible that an aspect of toxicity, besides covalent binding, that DAS inhibits, but NAC does not is responsible for triggering hsp induction.

In our mouse model, NAC did not affect APAP binding to protein when administered 1 or 3 hours after the APAP dose with a moderate decrease in binding observed in mice administered NAC concurrently with APAP. This conflicts with a past

report showing that NAC did inhibit binding of APAP when administered one hour after the APAP dose (Corcoran et al., 1985). In that study they referenced the radiolabeled APAP binding data to mouse body weight instead of the actual amount of liver protein present. They concluded that this prevented a falsely deflated value of covalent binding in mice administered APAP alone since influx of fluid and extrahepatic protein during APAP exposure would not affect the binding value; whereas, basing the binding value on the amount of protein present would include the extrahepatic protein that infiltrated the liver and was not available for covalent binding since the infiltration occurs after maximal covalent binding of APAP. However, that study provided no evidence of the alteration in protein levels in the liver after APAP exposure to back up their assumptions. Using more up to date techniques (i.e., Western blots) we have shown that NAC does not affect APAP binding when administered 1 or 3 hours after the APAP dose and only moderately affects binding when administered simultaneously. Since liver homogenate was loaded on a protein amount basis it was necessary to ensure that influx of extrahepatic protein did not cause a decrease in the amount of liver protein loaded. The level of three proteins (i.e., 55 and 100 kDa ABP, and actin) were not affected by APAP exposure indicating that influx of extrahepatic protein represented a small percentage of the total liver protein loaded (see Figure 6-3). In our model and at the dose of APAP used, basing binding data relative to the amount of protein is acceptable and no correction for edema and influx of extrahepatic protein is necessary since it contributes an insignificant amount, if any at all, compared to total liver protein.

It is remarkable that NAC did not prevent APAP binding when administered 1 or 3 hours after the dose and produced only a moderate decrease in binding when administered simultaneously since it is commonly accepted that NAC can act as a precursor to GSH to maintain the levels of GSH after APAP exposure and detoxify the reactive metabolite of APAP (Corcoran and Wong, 1986; Lauterburg et al., 1983). Our data argue against this mechanism of NAC action in preventing APAP hepatotoxicity, at least when administered 1

hour after the dose, since binding was not inhibited by NAC which would be expected if NAC produced excess GSH which scavenged the APAP reactive metabolite. When administered at the same time as APAP, NAC probably does directly scavenge the APAP reactive metabolite since binding was slightly decreased in this treatment group. Past reports indicated that NAC formed an adduct with APAP, but it was much lower in amount compared to the APAP-GSH adduct; however, our GSH data back up the conclusion that NAC did not work by increasing GSH levels when administered with APAP since NAC did not have a profound effect on GSH levels in that treatment group. This was also the treatment group that had the greatest protection against APAP toxicity. Therefore, this group is the most representative of NAC's mechanism of protection since it separates the effect on NAC on GSH levels from its protective effects. It seems more likely that NAC prevents or reverses APAP toxicity simply by supplying reduced sulfhydryl groups to the cell that can participate in disulfide exchange rather than preventing or reversing APAP arylation of protein. This point is highlighted by two other observations. Dithiothreitol can protect against APAP toxicity in isolated mouse and hamster hepatocytes after maximal covalent binding has occurred (Tee et al., 1986; Harman and Self, 1986), suggesting that simply providing sulfhydryl reducing equivalents is sufficient to prevent APAP hepatotoxicity even in the presence of APAP binding. Second, NAC can provide dramatic protection in some mammalian species, such as humans, when administered up to 10 hours after the APAP dose (Prescott, 1983) and late treatment (i.e., a mean time to therapy of 53 hours post APAP ingestion) with NAC has been shown to decrease morbidity and mortality caused by APAP (Keays et al., 1991). Since maximal covalent binding of APAP occurs within several hours, NAC's protective effect obviously has nothing to do with preventing the binding of APAP to protein. It is possible that NAC reverses APAP binding, but the study by Corcoran et al. (1985) showed that NAC was incapable of cleaving the APAP-protein bond in liver homogenate; therefore, it probably does not reverse APAP binding in vivo which is further argued by the data presented in this report.

In conclusion, NAC did not inhibit the induction of hsp25 or hsp70i by APAP. In addition, NAC given at the same time as APAP did not prevent the depletion of GSH by APAP. These two observations suggest that protein adduction and sulfhydryl oxidation may play a key role in triggering hsp induction, at least during APAP exposure. This study further showed that covalent binding of APAP and toxicity could be separated indicating that covalent binding and GSH depletion alone are not sufficient to cause toxicity; however, they are probably sufficient to trigger hsp induction.

# CHAPTER 7
# CONCLUSIONS

## Conclusions

The data presented in this dissertation lend strong support for the hypothesis that hsp induction during hepatotoxicant exposure is a cellular defense mechanism to deal with proteotoxicity. Every hepatotoxicant tested (note: many toxicants not mentioned in this dissertation such as cadmium, diethylnitrosamine, allyl alcohol, chlorobenzene, and thioacetamide also caused hsp induction in the whole animal) was able to induce hsp synthesis in mouse liver at hepatotoxic doses. The induction was limited to hsp25 and hsp70i for each toxicant indicating that these hsps may be more sensitive to toxicant exposure and other stresses compared to the other hsps screened (i.e., hsp60, hsc70, and hsp90). In addition, significant liver damage had to occur before hsp induction was observed. Non-hepatotoxic doses of each toxicant were tested, but failed to induce any of the hsps screened. In the HepG2 cell line, only 4 out of 6 toxicants caused induction of hsp70i. Additional experiments measuring the metabolism of the toxicants indicated that the lack of hsp70i induction by carbon tetrachloride and cocaine may have been an artifact of the model cell line used since neither toxicant was metabolized into reactive intermediates that covalently bound protein in HepG2 cells in contrast to their bioactivation in vivo. This preliminary screening provided a strong foundation on which to build subsequent experiments since it showed that hepatotoxicants that cause damage through a vast array of mechanisms were able to induce hsps in the target organ at necrogenic doses.

Immunohistochemical detection of hsp25 and hsp70i accumulation in mouse liver after hepatotoxicant exposure provided a unique perspective on how hsp induction is

regulated by toxicants and what purpose they may serve during toxicant exposure. Hsp25 accumulation after APAP or bromobenzene showed a very interesting pattern of induction. Accumulation was predominantly in hepatocytes on the periphery and surrounding the toxicant-induced lesions 24 hours after exposure to either of these toxicants. Hsp70i induction also had a tendency to be induced on the periphery of the lesions, but not as intensely as for hsp25. In contrast, hsp25 and hsp70i induction after carbon tetrachloride or cocaine exposure was restricted to hepatocytes within the toxicant-induced lesions. Accumulation of both hsps was also uniform throughout the lesions.

Immunohistochemical analysis of toxicant adduction of protein using antibodies that recognized APAP or cocaine provided a powerful tool to determine the correlation between toxicant adduction of protein and hsp induction. This is a fundamental question since denaturation of protein is believed to be sufficient to trigger hsp induction. If this is also the case for toxicant-adducted protein, then it would be expected that only cells that contain toxicant adducts would also have hsp induction. The presented data fit this model very well. Hsp induction after APAP or cocaine exposure was limited to hepatocytes that contained covalently bound toxicant. If a hepatocyte did not contain toxicant adduct it also did not have hsp accumulation. However, there are cases after APAP exposure in which some hepatocytes had APAP adducts and hsp25 induction without simultaneous hsp70i induction, indicating that toxicant adduction of protein may not be the only trigger of induction for some hsps.

Numerous studies have shown that elevated levels of hsps are correlated with protection from a variety of adverse stimuli and more recently it has been shown that hsps alone are capable of providing protection. The ability of elevated levels of hsps to provide protection against toxicant exposure in cells and the whole animal was measured to determine if hsps can provide protection against the type of damage a variety of toxicants cause. In both cells and the whole animal, elevated levels of hsps produced through hyperthermia (cells) or amphetamine-pretreatment (whole animal) provided protection

against some, but not all hepatotoxicants. In HepG2 cells, a strong correlation between the ability of a toxicant to induce hsp70i and the ability of a prior sub-LHS to provide protection against that toxicant was observed. The greater a toxicant induced hsp70i, the more a prior sub-LHS lowered the cytotoxicity of that toxicant. This may be representative of the type of cellular damage that causes hsp induction and the ability of hsps to repair that damage.

Even though all four hepatotoxicants screened in the whole animal were able to induce hsp25 and hsp70i, amphetamine pretreatment only provided protection against two of the toxicants. Therefore, the correlation that existed in cells did not pertain to the whole animal. This must be interpreted with caution since amphetamine might be mechanistically different than hyperthermia in providing protection; however, this seems unlikely since amphetamine is believed to cause hsp induction through its hyperthermic effects. There was a strong correlation between the pattern of hsp25 accumulation caused by a given toxicant and the ability of amphetamine to provide protection against that toxicant. APAP and bromobenzene both produced the "peripheral ring" pattern of hsp25 accumulation; whereas, carbon tetrachloride and cocaine produced a uniform induction of hsp25 that was restricted to damaged hepatocytes. It is possible that the pattern of hsp25 accumulation is representative of a protective pattern of hsp25 during toxicant exposure.

Several pieces of evidence presented in this dissertation lend strong support for the ability of toxicant adducted protein to induce hsps; however, this is probably not the only trigger of hsp induction. Other mechanisms of toxicant action may contribute to hsp induction which is suggested by the binding of AMAP to protein, but its failure to induce any hsp examined. There are several possible explanations of why AMAP failed to cause hsp induction which were mentioned in Chapter 4; however, since AMAP covalently bound protein, but did not trigger hsp induction it appears that toxicant adduction of protein alone may not be sufficient to trigger hsp induction in some circumstances.

To gain more insight into the role of toxicant adduction of protein in triggering hsp induction, the ability of APAP to induce hsp accumulation without concurrent toxicity was examined. As mentioned before, a non-hepatotoxic doses of APAP failed to cause hsp induction. However, at non-hepatotoxic doses, APAP fails to covalently bind protein since the reactive metabolites are scavenged by GSH before they can bind cellular macromolecules. Therefore, lower doses do not provide an answer as to whether toxicant adducted protein, without concurrent toxicity, is sufficient to cause hsp induction. NAC provided a unique pharmacological means to eliminate APAP-induced hepatotoxicity without dramatically affecting the arylation of protein. These studies revealed that toxicant adducted protein alone was sufficient to trigger hsp induction without ensuing toxicity. Other aspects of APAP hepatotoxicity not inhibited by NAC, besides covalent binding, can not be ruled out, however, as a trigger for hsp induction. This is represented by the effect of NAC on APAP-induced GSH depletion. NAC did not prevent the depletion of GSH by APAP when given at the same time as APAP (i.e., when NAC was most protective) indicating that oxidation of sulfhydryl groups may play some role in triggering hsp induction.

In conclusion, a variety of hepatotoxicants induced hsp25 and hsp70i expression in mouse liver when administered at a necrogenic dose. Immunohistochemical detection of hsp levels in liver revealed that hsp induction was restricted to the lesion for some toxicants (i.e., carbon tetrachloride and cocaine) and was found to be predominantly on the periphery and surrounding the lesions for others (i.e., bromobenzene and APAP). In addition, a strong correlation existed between the intralobular pattern of cocaine and APAP adduction of protein and the pattern of hsp accumulation. This suggested that toxicant-adduction of protein may play a role in triggering hsp induction. Elevated levels of hsps produced by an amphetamine pretreatment protected against APAP and bromobenzene toxicity without affecting the potency of carbon tetrachloride or cocaine. Bromobenzene and APAP were also the two toxicants that produced the "peripheral ring" pattern of hsp25 accumulation,

suggesting that hsp25 induction during exposure to these two toxicants may be a mechanism to provide protection against the damage caused by these toxicants. Finally, NAC treatment did not dramatically alter the binding of APAP to protein and did not inhibit APAP-induced hsp accumulation in the liver. NAC also eliminated APAP hepatotoxicity, suggesting that APAP arylation of protein may play a key role in triggering hsp induction.

LIST OF REFERENCES

Abe, K., Lee, T. H., Aoki, M., Nitta, Y., and Isoyama, S. (1993). Preferential expression of HSC70 heat shock mRNA in gerbil heart after transient brain ischemia. J. Mol. Cell Cardiol. 25, 1131-1135.

Abe, T., Konishi, T., Katoh, Takahiko, Hirano, H., Matsukuma, K., Kashimura, M., and Higashi, K. (1994). Induction of heat shock 70 mRNA by cadmium is mediated by glutathione suppressive and non-supressive triggers. Biochimica et Biophysica Acta 1201, 29-36.

Abravaya, K., Myers, M. P., Murphy, S. P., and Morimoto, R. I. (1992). The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. Genes Dev. 6, 1153-1164.

Ambre, J., and Alexander, M. (1977). Liver toxicity after acetaminophen ingestion: inadequacy of the dose estimate as an index of risk. J. Am. Med. Assoc. 238, 500-501.

Amin, J., Ananthan, J., and Voellmy, R. (1988). Key features of heat shock regulatory elements. Molecular and Cellular Biology 8: 3761-3769.

Ananthan, J., Goldber, A. L., and Voellmy, R. (1986). Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. Science 232, 522-524.

Anderson, N. L., Giere, F. A., Nance, S. L., Gemmell, M. A., Tollaksen, S. L. and Anderson, N. G. (1987). Effects of toxic agents at the protein level: quantitative measurement of 213 mouse liver proteins following xenobiotic treatment. Fund. Appl. Toxicol. 8, 39-50.

Baler, R. (1992). Ph. D. Thesis: Purification, partial characterization and regulation of human heat shock transcription factor. University of Miami.

Baler, R., Dahl, G., and Voellmy, R. (1993). Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1. Mol. Cell. Biol. 13, 2486-2496.

Baler, R., Welch, W. J., and Voellmy, R. (1992). Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. J. Cell Biol. 117, 1151-1159.

Bardella L, Schiaffonati L, Cairo G, Bernelli-Zazzera A. (1987). Heat-shock proteins and mRNA's in liver and hepatoma. British Journal of Cancer 55, 643-645.

Bartolone, J. B., Birge, R. B., Bulera, S. J., Bruno, M. K., Nishanian, E. V., Cohen, S. D., and Khairallah, E. A. (1992). Purification, antibody production, and partial

amino acid sequence of the 58-kDa acetaminophen-binding liver proteins. Toxicol. Appl. Pharmacol. 113, 19-29.

Bauman, J.W., Liu, J., and Klaassen, C.D. (1993). Production of metallothionein and heat-shock proteins in response to metals. Fundamental and Applied Toxicology 21: 15-22.

Beck, S.C., and De Maio, A.D. (1994). Stabilization of protein synthesis in thermotolerant cells during heat shock. The Journal of Biological Chemistry 269: 21803-21811.

Beckmann, R. P., Lovett, M., and Welch, W. J. (1992). Examining the function and regulation of hsp 70 in cells subjected to metabolic stress. The Journal of Cell Biology. 117: 1137-1150.

Beckmann, R.P., Mizzen, L.A., and Welch, W.J. (1990). Interaction of hsp 70 with newly synthesized proteins: implications for protein folding and assembly. Science 248: 850-854.

Belisario, M. A., Arena, A. R., Pecce, R., Borgia, R., Staiano, N., and Lorenzo, F. D. (1991). Effect of enzyme inducers on metabolism of 1-nitropyrene in human hepatoma cell line HepG2. Chem. Biol. Interactions. 78, 253-268.

Bergmeyer, H. U., Scheibe, P., Wahlefeld, A. W. (1978). Optimization of methods for aspartate aminotransferase and alanine aminotransferase. Clin. Chem. 24, 58-73.

Bernelli-Zazzera, A., Cairo, G., Schiaffonati, L., and Tacchini, L. (1992). Stress proteins and reperfusion stress in the liver. Annals New York Academy of Sciences 663: 120-124.

Birge, R. B., Bartolone, J. B., Emeigh-Hart, S. G., Nishanian, E. V., Tyson, C. A., Khairallah, E. A., and Cohen, S. D. (1990). Acetaminophen hepatotoxicity: correspondence of selective protein arylation in human and mouse liver in vitro, in culture, and in vivo. Toxicol. Appl. Pharmacol. 105, 472-482.

Blake, M. J., Gershon, D., Fargnoli, J., and Holbrook, N. J. (1990). Discordant expression of heat shock protein mRNA's in tissues of heat-stressed rats. The Journal of Biological Chemistry, 265, 15275-15279.

Blom, A., Harder W., and Martin, A. (1992). Unique and overlapping pollutant stress proteins of Escherichia coli. Appl. Environ. Microbiol. 58, 331-338.

Boelsterli, U. A., and Goldlin, C. (1991). Biomechanisms of cocaine-induced hepatocyte injury mediated by the formation of reactive metabolites. Arch. Toxicol. 65, 351-360.

Boyer, T. D., and Rouff, S. L. (1971). Acetaminophen-induced hepatic necrosis and renal failure. J. Am. Med. Assoc. 218, 440-441.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brown, C.R., Martin, R.L., Hansen, W.J., Beckmann, R.P., and Welch, W.J. (1993). The constitutive and stress inducible forms of hsp 70 exhibit functional similarities

and interact with one another in an ATP-dependent fashion. The Journal of Cell Biology 120: 1101-1112.

Bulera, S. J., Birge, R. B., Cohen, S. D., and Khairallah, E. A. (1995). Identification of the mouse liver 44-kDa acetaminophen-binding protein as a subunit of glutamine synthetase. Toxicol. Appl. Pharmacol. 134, 313-320.

Burel, C., Mezger, V., Pinto, M., Rallu, M., Trigon, S., and Morange, M. (1992). Mammalian heat shock protein families. Expression and functions. Experientia 48: 629-634.

Bushnell, P. J., and Gordon, C. J. (1987). Effects of d-amphetamine on behavioral and autonomic thermoregulation in mice. Pharmacology Biochemistry and Behavior 27, 431-435.

Callaway, C. W., and Clark, R. F. (1994). Hyperthermia in psychostimulant overdose. Annals of Emergency Medicine 24, 68-75.

Casini, A. F., Pompella, A., and Comporti, M. (1985). Liver glutathione depletion induced by bromobenzene, iodobenzene, and diethylmaleate poisoning and its relation to lipid peroxidation and necrosis. Am. J. Pathol. 118, 225-237.

Chen, Q., Kefu, Y., and Stevens, J. L. (1992). Regulation of the cellular stress response by reactive electrophiles. Journal Biol. Chem. 267, 24322-24327.

Chirico, W.J. (1992). Dissociation of complexes between 70 kDa stress proteins and presecretory proteins is facilitated by a cytosolic factor. Biochemical and Biophysical Research Communications 189: 1150-1156.

Chomczynski, P., Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156-159.

Ciooca, D.R., Oesterreich, S., Chamness, G.C., McGuire, W.L., Fuqua, S.A. (1993). Biological and clinical implications of heat shock protein 27000 (hsp27): a review. Journal of the National Cancer Institute 85: 1558-1570.

Collison, M.W., Beidler, D., Grimm, L.M., and Thomas, J.A. (1986). A comparison of protein S-thiolation (protein mixed-disulfide formation) in heart cells treated with t-butyl hydroperoxide or diamide. Biochimica et Biophysica Acta 885: 58-67.

Corcoran, G. B., and Wong, B. K. (1986). Role of glutathione in prevention of acetaminophen-induced hepatotoxicity by N-acetyl-L-cysteine in vivo: Studies with N-acetyl-D-cysteine in mice. J. Pharmacol. Exp. Ther. 238, 54-61.

Corcoran, G. B., Mitchell, J. R., Vaishnav, Y. N., and Horning, E. C. (1980). Evidence that acetaminophen and N-hydroxyacetaminophen form a common arylating intermediate, N-acetyl-p-benzoquinoneimine. Mol. Pharmacol. 18, 536-542.

Corcoran, G. B., Racz, W. J., Smith, C. V., and Mitchell, J. R. (1985). Effects of N-acetylcysteine on acetaminophen covalent binding and hepatic necrosis in mice. J. Pharmacology Experimental Therapeutics 232, 864-872.

Craig, E. A., Baxter, B. K., Becker, J., Halladay, J., and Ziegelhoffer, T. (1994). Cytosolic hsp70s of Saccharomyces cerevisiae: Roles in protein synthesis, protein

translocation, proteolysis, and regulation. In Biology of Heat Shock Proteins and Molecular Chaperones. (R. Morimoto, A. Tissieres, and C. Georgopoulos, Eds.), pp. 31-52. Cold Spring Harbor Laboratory Press, New York.

Currie, R. W., Karmazyn, M., Kloc, M., and Mailer, K. (1988). Heat-shock response is associated with enhanced postischemic ventricular recovery. Circulation Research 63, 543-549.

Dahlin, D. C., Miwa, G. T., Lu, A. Y. H., and Nelson, S. D. (1984). N-Acetyl-p-benzoquionone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. Proc. Natl. Acad. Sci. 81, 1327-1331.

De Maio, A., Beck, S. C., and Buchman, T. G. (1993). Heat shock gene expression and development of translational thermotolerance in human hepatoblastoma cells. Circ. Shock 40, 177-186.

De Maio, A., Beck, S. C., and Buchman, T. G. (1993). Induction of translational thermotolerance in liver of thermally stressed rats. Eur. J. Biochem. 218, 413-420.

DeRoos, F. J., and Hoffman, R. S. (1996). Drug-induced hepatotoxicity. N. Engl. J. Med. 334, 863.

Doostdar, H., Grant, M. H., Melvin, W. T., Wolf, C. R., and Burke, M. D. (1993). The effects of inducing agents on cytochrome P450 and UDP-glucuronyltransferase activities in human HepG2 hepatoma cells. Biochem. Pharmacol. 46, 629-635.

Ellman, G. L. (1959). Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82, 70-77.

Estrela, J. M., Saez, G. T., Such, L., and Vina, J. (1983). The effect of cysteine and N-acetylcysteine on rat liver glutathione (GSH). Biochemical Pharmacology 32, 3483-3485.

Evans, M. A. (1983). Role of protein binding in cocaine-induced hepatic necrosis. J. Pharmacol. Exp. Ther. 224, 73-79.

Feige, U., and Mollenhauer, J. (1992). Heat shock proteins. Experientia 48: 621-622.

Fernandes, M., O'Brien, T., Lis, J.T. (1994). Structure and regulation of heat shock gene promoters. In Biology of Heat Shock Proteins and Molecular Chaperones. (R. Morimoto, A. Tissieres, C. Georgopoulos, Eds.), pp. 375-393. Cold Spring Harbor Laboratory Press, New York.

Flynn, G. C., Chappell, T. G., and Rothman, J. E. (1989). Peptide binding and release by proteins implicated as catalysts of protein assembly. Science 245, 385-390.

Gerber, J. G., MacDonald, J. S., Harbison, R. D., Villeneuve, J. P., Wood, A. J. J., and Nies, A. S. (1977). Effect of N-acetylcysteine on hepatic covalent binding of paracetamol (acetaminophen). Lancet 1, 657-658.

Goering, P. L., Fisher, B. R., and Kish, C. L. (1993). Stress protein synthesis induced in rat liver by cadmium precedes hepatotoxicity. Toxicol. Appl. Pharmacol. 122, 139-148.

Goff, S. A., and Goldberg, A. L. (1985). Production of abnormal proteins in E. coli stimulates transcription of lon and other heat shock genes. Cell 41, 587-595.

Goodman, J. H., and Sloviter, R. S. (1993). Cocaine neurotoxicity and altered neuropeptide Y immunoreactivity in the rat hippocampus; a silver degeneration and immunocytochemical study. Brain Research 616, 263-272.

Grimm, L.M., Collison, M.W., Fisher, R.A., and Thomas, J.A. (1985). Protein mixed-disulfides in cardiac cells. S-Thiolation of soluble proteins in response to diamide. Biochimica et Biophysica Acta 844: 50-54.

Hahn, G. M., and Li, G. C. (1982). Thermotolerance and heat shock proteins in mammalian cells. Radiation Research 92, 452-457.

Hahn, G. M., and Li, G. C. (1990). Thermotolerance, thermoresistance, and thermosensitization. In Stress Proteins in Biology and Medicine (R. Morimoto, A. Tissieres, C. Georgopoulos, Eds.), pp. 79-101. Cold Spring Harbor Laboratory Press, New York.

Hanzlik, R. P., Weller, P. E., Narashimhan, N., and Buben, J. A. (1989). Metabolic activation and protein covalent binding chemistry of bromobenzene and derivatives. In Xenobiotic Metabolism and Disposition. (R. Kato, Ed.), pp. 367-374. Taylor and Francis, London.

Harman, A. W., and Self, G. (1986). Comparison of the protective effects of N-acetylcysteine, 2-mercaptopropionylglycine and dithiothreitol against acetaminophen toxicity in mouse hepatocytes. Toxicology 41, 83-93.

Hightower, L. E. (1991). Heat shock, stress proteins, chaperones, and proteotoxicity. Cell 66, 191-197.

Hightower, L. E., Sadis, S. E., and Takenaka, I. M. (1994). Interactions of vertebrate hsc70 and hsp70 with unfolded proteins and peptides. In The Biology of Heat Shock Proteins and Molecular Chaperones. (R. Morimoto, A. Tissieres, C. Georgopoulos, Eds.), pp. 179-207. Cold Spring Harbor Laboratory Press, New York.

Hinson, J. A. (1980). Biochemical toxicology of acetaminophen. Rev. Biochem. Toxicol. 2, 103-130.

Hotchkiss, R., Nunnally, I., Lindquist, S., Taulien, J., Perdrizet, G., and Karl, I. (1993). Hyperthermia protects mice against the lethal effects of endotoxin. American Journal of Physiology 265, R1447-R1457.

Huang, L. E., Zhang, H., Bae, S. W., and Liu, A. Y. C. (1994). Thiol reducing reagents inhibit the heat shock response. J. Biol. Chem. 269, 30718-30725.

Jaattela, M., and Wissing, D. (1992). Emerging role of heat shock proteins in biology and medicine. Annals of Medicine 24, 249-258.

Jacobson, K. B., and Turner, J. E. (1980). The interaction of cadmium and certain other metal ions with proteins and nucleic acids. Toxicology 16, 1-37.

James, R.C., Harbison, R.D., and Roberts, S.M. (1993). Phenylpropanolamine potentiation of acetaminophen-induced hepatotoxicity: Evidence for a glutathione-dependent mechanism. Toxicol. Apply. Pharmacol. 118, 159-168.

Joslin, G., Hafeez, W., and Perlmutter, D. H. (1991). Expression of stress proteins in human mononuclear phagocytes. The Journal of Immunology 147, 1614-1620.

Kampinga, H. H., Brunsting, J. F., Stege, G. J. J., Burgman, P. W. J. J., and Konings, A. W. T. (1995). Thermal protein denaturation and protein aggregation in cells made thermotolerant by various chemicals: role of heat shock proteins. Exp. Cell Res. 219, 536-546.

Kampinga, H. H., Brunsting, J. F., Stege, G. J. J., Burgman, P. W. J. J., and Konings, A. W. T. (1995). Thermal protein denaturation and protein aggregation in cells made thermotolerant by various chemicals: role of heat shock proteins. Exp. Cell Res. 219, 536-546.

Kanekal, S., Fraiser, L., and Kehrer, J. P. (1992). Pharmacokinetics, metabolic activation, and lung toxicity of cyclophosphamide in C57/B16 and ICR mice. Toxicol. Appl. Pharmacol. 114, 1-8.

Keays, R., Harrison, P. M., Wendon, J. A., Forbes, A., Gove, C., Alexander, G. J. M., and Williams, R. (1991). Intravenous acetylcysteine in paracetamol induced fulminant hepatic failure: a prospective controlled trial. British Medical Journal 303, 1026-1029.

Kim, Y-J., Shuman, J., Sette, M., and Przybyla, A. (1983). Arsenate induces stress proteins in cultured rat myoblasts. J. Cell. Biol. 96, 393-400.

Klemenz, R., Andres, A. C., Frohli, E., Schafer, R., and Aoyama, A. (1993). Expression of the murine small heat shock proteins hsp25 and aB Crystallin in the absence of stress. J. Cell. Biol. 120, 639-645.

Kloss, M. W., Rosen, G. M., Rauckman, E. J. (1983). Evidence of enhanced in vivo lipid peroxidation after acute cocaine adminstration. Toxicol. Lett. 15, 65-70.

Laemmli, V. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lalitha, T., Kerem, D., and Yannai, S. (1990). Effect of N-acetyl-cysteine, D-penicillamine and buthionine sulfoximine on glutathione levels and CNS oxygen toxicity in rats. Pharmacology and Toxicology 66, 56-61.

Lauterberg, B. H., Corcoran, G. B., and Mitchell, J. R. (1983). Mechanism of action of N-acetylcysteine in the protection against the hepatotoxicity of acetaminophen in rats in vivo. J. Clin. Invest. 71, 980-991.

Lee, K. J., and Hahn, G. M. (1988). Abnormal proteins as the trigger for the induction of stress responses: heat, diamide, and sodium arsenite. Journal of Cellular Physiology 136, 411-420.

Lee, S. S. T., Buters, J. T. M., Pineau, T., Fernandez-Salguero, P., and Gonzalez, F. J. (1996). Role of CYP2E1 in the hepatotoxicity of acetaminophen. J. Biol. Chem. 271, 12063-12067.

Lee, Y.J., and Dewey, W.C. (1988). Thermotolerance induced by heat, sodium arsenite, or puromycin: its inhibition and differences between 43°C and 45°C. Journal of Cellular Physiology 135: 397-406.

Levinson, W., Oppermann, H., and Jackson, J. (1980). Transition series metals and sulfhydryl reagents induce the synthesis of four proteins in eukaryotic cells. Biochim. Biophys. Acta. 606, 170-180.

Li, G. C., and Werb, Z. (1982). Correlation between synthesis of heat shock proteins and development of thermotolerance in chinese hamster fibroblasts. Proc. Natl. Acad. Sci. 79, 3218-3222.

Li, G. C., Li, L., Liu, Y. K., Mak, J. Y., Chen, L., and Lee, W. M. F. (1991). Thermal response of rat fibroblasts stably transfected with the human 70-kDa heat shock protein-encoding gene. Proc. Natl. Acad. Sci. 88, 1681-1685.

Lin, W.Q., VanDyke, R.A., Marsh, H.M., and Trudell, J.R. (1994). Nuclear translocation of heat shock protein 72 in liver cells of halothane-exposed rats. Biochemical and Biophysical Research Communications 199: 647-652.

Lindamood, C. (1991). Xenobiotic. In: Hepatotoxicology. (Meeks et al., eds.), pp. 139-180. CRC Press, Boston.

Lindquist, S., and Craig, E. A. (1988). The heat-shock proteins. Annu. Rev. Genet. 22, 631-677.

Lu, D., Das, D. K. (1993). Induction of differential heat shock gene expression in heart, lung, liver, brain, and kidney by a sympathomimetic drug, amphetamine. Biochem. Biophys. Res. Commun. 192, 808-812.

Lumpkin, C.K., Henle, K.J., Sammartino, G., Nolen, G.T., and Taylor, J.M. (1988). Expression of thermotolerance following microinjection of glutathione disulfide. Radiation Research 115: 202-210.

Manautou, J. E., Emeigh-Hart, S. G., Khairallah, E. A., and Cohen, S. D. (1996). Protection against acetaminophen hepatotoxicity by a single dose of clofibrate: effects on selective protein arylation and glutathione depletion. Fund. Appl. Toxicol. 29, 229-237.

Marzella, L., and Trump, B.F. (1991). Pathology of the liver: functional and structural alterations of hepatocyte organelles induced by cell injury. In Hepatotoxicology. (T. Meeks, Ed.), pp. 93-137. CRC Press, Boston.

Matthews, A. M., Roberts, D. W., Hinson, J. A., and Pumford, N. R. (1996). Acetaminophen-induced hepatotoxicity: Analysis of total vs. specific binding to cysteine. Drug Metabolism and Disposition 24, 1192-1196.

Matthews, E. K., Flaherty, C., and Smith, W. H. T. (1993). Photodynamic action of aluminium phthalocyanine tetrasulphonate ($AlPcS_4$) on smooth muscle: effects of thiols and a cyclic GMP analogue. Br. J. Pharmacol. 110, 1248-1254.

McLellan, L. 1., Lewis, A. D., Hall, D. J., Ansell, J. D., and Wolf, C. R. (1995). Uptake and distribution of N-acetylcysteine in mice: tissue-specific effects on glutathione concentrations. Carcinogenesis 16, 2099-2106.

Mirkes, P. E., Doggett, B., and Cornel, L. (1994). Induction of a heat shock response (HSP 72) in rat embryos exposed to selected chemical teratogens. Teratology 49, 135-142.

Morimoto, R. 1., Jurivich, D. A., Kroeger, P. E., Mathur, S. K., Murphy, S. P., Nakai, A., Sarge, K., Abravaya, K., and Sistonen, L. T. (1994). Regulation of heat shock gene transcription by a family of heat shock factors. In Biology of Heat Shock Proteins and Molecular Chaperones. (R. Morimoto, A. Tissieres, C. Georgopoulos, Eds.), pp. 417-455. Cold Spring Harbor Laboratory Press, New York.

Myers, M. W., Quirk, W. S., Rizk, S. S., Miller, J. M., and Altschuler, R. A. (1992). Expression of the major mammalian stress protein in the rat cochlea following transient ischemia. Laryngoscope 102, 981-987.

Myers, T. G., Dietz, E. C., Leigh-Anderson, N., Khairallah, E. A., Cohen, S. D., and Nelson, S. D. (1995). A comparative study of mouse liver proteins arylated by reactive metabolites of acetaminophen and its nonhepatotoxic regioisomer, 3'-hydroxyacetanilide. Chem. Res. Toxicol. 8, 403-413.

Neuman, M. G., Koren, G., and Tiribelli, C. (1993). In Vitro assessment of the ethanol-induced hepatotoxicity on HepG2 cell line. Biochem. Biophys. Res. Commun. 197, 932-941.

Nover, L. (1991). Heat Shock Response. CRC Press, Boca Raton, FL.

Nowak, T. S. (1988). Effects of amphetamine on protein synthesis and energy metabolism in mouse brain: role of drug-induced hyperthermia. Journal of Neurochemistry 50, 285-294.

Osterman-Golkar, S., and Bergmark, E. (1988). Alkylation of haemoglobin, plasma proteins and DNA in the mouse by diethylnitrosamine. Carcinogenesis 9, 1915-1917.

Ovelgonne, H. H., Wiegant, F. A. C., Souren, J. E. M., VanRijn, H., and VanWijk, R. (1995). Enhancement of the stress response by low concentrations of arsenite in arsenite-pretreated Reuber H35 hepatoma cells. Toxicol. Appl. Pharmacol. 132, 146-155.

Palleros, D. R., Welch, W. J., and Fink, A. L. (1991). Interaction of hsp70 with unfolded proteins: effects of temperature and nucleotides on the kinetics of binding. Proc. Natl. Acad. Sci. 88, 5719-5723.

Parag, H.A., Raboy, B., and Kulka, R.G. (1987). Effect of heat shock on protein degradation in mammalian cells: involvement of the ubiquitin system. The EMBO Journal 6: 55-61.

Parsell, D. A., and Lindquist, S. (1994). Heat shock proteins and stress tolerance. In Biology of Heat Shock Proteins and Molecular Chaperones. (R. Morimoto, A.

Tissieres, and C. Georgopoulos, Eds.), pp. 457-494. Cold Spring Harbor Laboratory Press, New York.

Petersen, R., and Lindquist, S. (1988). The Drosophila hsp70 message is rapidly degraded at normal temperatures and stabilized by heat shock. Gene 72: 161-168.

Plaa, G. L. (1993). Toxic responses of the liver. In Toxicology: The Basic Science of Poisons. (C. Klaassen, Ed.), pp. 334-353. McGraw-Hill, New York.

Powell, C. J., Connolly, A. K., and Charles, S. J. (1991). Shifting necrosis: butylated hydroxytoluene (BHT) and phenobarbital move cocaine-induced hepatic necrosis across the lobule. Toxicol. Letters 55, 171-178.

Prescott, L. F. (1983). Paracetamol overdosage: Pharmacological considerations and clinical management. Drugs 25, 290-314.

Ramsay, R. R., Rashed, M. S., and Nelson, S. D. (1989). In Vitro effects of acetaminophen metabolites and analogs on the respiration of mouse liver mitochondria. Arch. Biochem. Biophys. 273, 449-457.

Rashed, M. S., and Nelson, S. D. (1989). Characterization of glutathione conjugates of reactive metabolites of 3'-hydroxyacetanilide, a nonhepatotoxic positional isomer of acetaminophen. Chem. Res. Toxicol. 2, 41-45.

Rashed, M. S., Myers, T. G., and Nelson, S. D. (1990). Hepatic protein arylation, glutathione depletion, and metabolite profies of acetaminophen and a non-hepatotoxic regioisomer, 3'-hydroxyacetanilide, in the mouse. Drug Metabolism and Disposition 18, 765-770.

Recknagel, R. O., Glende, E. A., Dolak, J. A., and Waller, R. L. (1989). Mechanisms of carbon tetrachloride toxicity. Pharmac. Ther. 43, 139-154.

Riabowol, K. T., Mizzen, L. A., and Welch, W. J. (1988). Heat shock is lethal to fibroblasts microinjected with antibodies against hsp70. Science 242, 433-436.

Ritosa, F. (1962). A new puffing pattern induced by temperature shock and DNP in Drosophila. Experimentia 18: 571-573.

Roberts, S. A., Price, V. F., and Jollow, D. J. (1990). Acetaminophen structure-toxicity studies: in vivo covalent binding of a nonhepatotoxic analog, 3-hydroxyacetanilide. Toxicol. Appl. Pharmacol. 105, 195-208.

Roberts, S. M., Harbison, R. D., and James, R. C. (1995). Mechanistic studies on the potentiation of carbon tetrachloride hepatotoxicity by methamphetamine. Toxicology 97, 49-57.

Roberts, S. M., James, R. C., and Harbison, R. D. (1992). Cocaine-induced hepatotoxicity. In: Cocaine. Pharmacology, Physiology, and Clinical Strategies. (J. Lakoski, M. Galloway, F. White, Eds.), pp. 15-33. CRC Press, Boca Raton.

Roberts, S. M., Voellmy, R. M., and Salminen, W. F. (1996). Immunohistochemical localization of hsp25 and hsp70 induction in murine liver after acute treatment with carbon tetrachloride or bromobenzene. The Toxicologist 30, 82.

Roberts, S.M., DeMott, R., and James, R.C. (1997) Adrenergic modulation of hepatotoxicity. Drug Metab. Rev. (in press)

Roth, L., Harbison, R. D., James, R. C., Tobin, T., and Roberts, S. M. (1992). Cocaine hepatotoxicity: influence of hepatic enzyme inducing and inhibiting agents on the site of necrosis. Hepatology 15, 934-940.

Rothman, J.E. (1989). Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. Cell 59: 591-601.

Saad, S., Kanai, M., Awane, M., Yamamoto, Y., Morimoto, T., Isselhard, W., Minor, T., Troidl, H., Ozawa, K., and Yamaoka, Y. (1995). Protective effect of heat shock pretreatment with heat shock protein induction before hepatic warm ischemic injury caused by Pringle's maneuver. Surgery 118, 510-516.

Salminen, W. F., Roberts, S. M., Fenna, M., and Voellmy, R. M. (1997). Heat shock protein induction in murine liver after acute treatment with cocaine. Hepatology (In Press).

Salminen, W. F., Voellmy, R. M., Roberts, S. M. (1996). Induction of hsp70 in HepG2 cells in response to hepatotoxicants. Toxicol. Appl. Pharmacol. 141, 117-123.

Sanchez, Y., Taulien, J., Borkovich, K.A., and Lindquist, S. (1992). Hsp104 is required for tolerance to many forms of stress. The EMBO Journal 11, 2357-2364.

Sanders, B. M. (1993). Stress proteins in aquatic organisms: an environmental perspective. Crit. Rev. Toxicol. 23, 49-75.

Sarge, K.D., Murphy, S.P., and Morimoto, R.I. (1993). Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. Molecular and Cellular Biology 13: 1392-1407.

Seale, T. W., Carney, J. M., Johnson, P., Rennert, O. M. (1985). Inheritance of amphetamine-induced thermoregulatory responses in inbred mice. Pharmacology Biochemistry and Behavior 23, 373-377.

Sedlak, J., and Lindasy, R. H. (1968). Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Analytical Biochemistry 25, 192-205.

Sipes, I. G., and Gandolfi, A. J. (1982). Bioactivation of aliphatic organohalogens: formation, detection, and relevance. In Toxicology of the Liver. (G. Plaa, and W. R. Hewitt, Eds.), pp 181-212. Raven Press, New York.

Steels, E. L., Watson, K., and Parsons, P. G. (1992). Relationships between thermotolerance, oxidative stress responses and induction of stress proteins in human tumor cell lines. Biochem. Pharmacol. 44, 2123-2129.

Tee, L. B. G., Boobis, A. R., Huggett, A. C., and Davies, D. S. (1986). Reversal of acetaminophen toxicity in isolated hamster hepatocytes by dithiothreitol. Toxicol. Appl. Pharmacol. 83, 294-314.

Theodorakis, N.G., Morimoto, R.I. (1987). Posttranscriptional regulation of hsp70 expression in human cells: effects of heat shock, inhibition of protein synthesis, and adenovirus infection on translation and mRNA stability. Molecular and Cellular Biology 7, 4357-4368.

Tirmenstein, M. A., and Nelson, S. D. (1989). Subcellular binding and effects on calcium homeostasis produced by acetaminophen and a nonhepatotoxic regioisomer, 3'-hydroxyacetanilide, in mouse liver. J. Biol. Chem. 264, 9814-9819.

Tirmenstein, M. A., and Nelson, S. D. (1990). Acetaminophen-induced oxidation of protein thiols. J. Biol. Chem. 265, 3059-3065.

Tirmenstein, M. A., and Nelson, S. D. (1991). Hepatotoxicity after 3'-hydroxyacetanilide administration to buthionine sulfoximine pretreated mice. Chem. Res. Toxicol. 4, 214-217.

Van Laack, R.L., Faustman, C., and Sebranek, J.G. (1993). Pork quality and the expression of stress protein hsp 70 in swine. Journal of Animal Science 71, 2958-2964.

VanDyke, R. A,, Mostafapour, S., Marsh, H. M., Li, Y., and Chopp, M. (1992). Immunocytochemical detection of the 72-kDa heat shock protein in halothane-induced hepatotoxicity in rats. Life Sci. 50, PL41-PL45.

Villar, J., Edelson, J. D., Post, M., Mullen, J. B., and Slutsky, A. S. (1993). Induction of heat stress proteins is associated with decreased mortality in an animal model of acute lung injury. Am. Rev. Respir. Dis. 147, 177-181.

Voellmy, R. (1994). Transduction of the stress signal and mechanisms of transcriptional regulation of heat shock/stress protein gene expression in higher eukaryotes. Crit. Rev. Eukaryotic Gene Expression 4, 357-401.

Voellmy, R. M., Salminen, W. F., Borgert, C. J., Westhouse, R. A., James, R. C., Harbison, R. D., and Roberts, S. M. (1994). Measurement of changes in liver levels of stress proteins following administration of selected hepatotoxicants in the mouse. The Toxicologist 14, 133.

Voellmy, R., Ahmed, A., Schiller, P., Bromley, P., and Rungger, D. (1985). Isolation and functional analysis of a human 70,000-dalton heat shock protein gene segment. Proc. Natl. Acad. Sci. 82, 4949-4953.

Welch, W. J. (1992). The role of heat-shock proteins as molecular chaperones. Curr. Opin. Cell. Biol. 3, 1033-1038.

Westwood, J. T., Clos, J., and Wu, C. (1992). Stress-induced oligomerization and chromosomal relocalization of heat-shock factor. Nature 353, 822-827.

Wiegant, F. A. C., Souren, J. E. M., van Rijn, J., van Wijk, R. (1994). Stressor-specific induction of heat shock proteins in rat hepatoma cells. Toxicology 94, 143-159.

Wilkinson, J. M., Pollard, I. (1993). Immunohistochemical localisation of the 25 kDa heat shock protein in unstressed rats: possible functional implications. The Anatomical Record 237, 453-457.

Wu, B., Hunt, C., and Morimoto, R. (1985). Structure and expression of the human gene encoding major heat shock protein HSP70. Mol. Cell. Biol. 5, 330-341.

Wu, C., Clos, J., Giorgi, G., Haroun, R. I., Kim, S. J., Rabindran, S. K., Westwood, J. T., Wisniewski, J., and Yim, G. (1994). Structure and regulation of heat shock transcription factor. In Biology of Heat Shock Proteins and Molecular Chaperones. (R. Morimoto, A. Tissieres, and C. Georgopoulos, Eds.), pp. 395-416. Cold Spring Harbor Laboratory Press, New York.

Yao, T., Esposti, S. D., Huang, L., Arnon, R., Spangenberger, A., and Zern, M. A. (1994). Inhibition of carbon tetrachloride-induced liver injury by liposomes containing vitamin E. Am. J. Physiol. 267, G476-G484.

Yehuda, S., and Wurtman, R. J. (1972). The effects of D-amphetamine and related drugs on colonic temperatures of rats kept at various ambient temperatures. Life Sciences 11, 851-859.

Zuo, J., Rungger, D., Voellmy, R. (1995). Multiple layers of regulation of human heat shock transcription factor 1. Mol. Cell. Biol. 15, 4319-4330.

## BIOGRAPHICAL SKETCH

The author was born in Buffalo, New York, to two wonderful parents, Bill and Carol Salminen, who raised their two sons to be free thinkers. At the age of four, his family moved to Rochester, New York, where he attended elementary through high school. After attending the University of California at Los Angeles (UCLA) for two years, he returned to Rochester where he earned his Bachelor of Arts degree in biology from the University of Rochester in 1992.

During his time at the University of Rochester, he worked in the Clinical Microbiology laboratory at Strong Memorial Hospital for two years. After obtaining his Bachelor of Arts degree, the author worked in a research laboratory at the Department of Immunology at the University of Rochester making transgenic mice. He started work towards his doctoral degree in the Department of Pharmacology and Therapeutics at the University of Florida in July of 1993. After obtaining his Ph.D. degree, he will be working for Exxon Biomedical Sciences, Inc. in New Jersey.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Stephen Roberts, Chair
Associate Professor of Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Susan Frost
Associate Professor of Biochemistry and Molecular Biology

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Margaret James
Professor of Medicinal Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Thomas Rowe
Associate Professor of Pharmacology and Therapeutics

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Kathleen Shiverick
Professor of Pharmacology and Therapeutics

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Dean, College of Medicine

Dean, Graduate School